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THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

ARTHUR HARDEN

AND

CHARLES ROBERT HARINGTON

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I. VITAMIN A AND CAROTENE.

IX. NOTES ON THE CONVERSION OF CAROTENE TO VITAMIN A IN THE COW.

By THOMAS MOORE.

From the Dunn Nutritional Laboratory, Cambridge University, and Medical Research Council.

(*Received November 7th, 1931.*)

In a previous paper [Moore, 1930] evidence has been advanced to show that carotene, or some part of it, suffers conversion in the rat to the colourless vitamin A. It is well known, however, that the degree of yellow pigmentation of the body-fat of animals varies considerably between different species. Thus the rat resembles the sheep and pig in possessing colourless body-fat, while man, the ox and the fowl have more or less pigmented fats. Before reaching any conclusion as to the general applicability of results obtained with the rat, therefore, it seemed desirable to examine the fate of carotene in animals of the latter group, in case any fundamental difference should be revealed.

In regard to the fowl, Capper, McKibbin and Prentice [1931] have already shown that the administration of carotene leads to the appearance of vitamin A in the liver oil, the fate of the pigment being in marked contrast with that of xanthophyll, which appears neither to suffer obvious change nor to be selectively absorbed in the liver, being found fairly evenly distributed throughout the body- and egg-yolk-fats, and endowing them with their characteristic colour [Palmer and Kempster, 1919]. The present experiments deal with the equally familiar case of the ox, which differs from the fowl in having carotene as the predominant pigment of the body-fat [Palmer and Eccles, 1914].

Since the experiments of Stephenson [1920] upon the vitamin A activity of decolorised butter-fat it has been assumed that the colourless vitamin A plays its part in bovine metabolism, and this finding has been amply confirmed by the recent work of Morton and Heilbron [1930], who have shown by spectroscopic means that both carotene and vitamin A are present in the unsaponifiable fraction of New Zealand butter-fat. It has also been found that whereas the feeding of carrots or green stuff to cows leads to increases both in the carotene content and vitamin A activity of the butter-fat, the feeding of cod-liver oil results only in increased vitamin A activity, the degree of yellow pigmentation remaining unchanged [Drummond *et al.*, 1924; Golding, Soames and Zilva, 1926]. These observations, which first seemed to support the view that no relation could exist between carotene and the vitamin, might now seem to

point to an incomplete conversion of carotene in the cow, or even to an interchangeability of the functions of the pigment and vitamin in this animal. The present work was carried out in the hope of shedding light on this question.

Experimental.

Since the large size of the cow makes it an unsuitable subject for such rigid experimental procedure as can be adopted in the case of the rat it is unavoidable that the evidence as to the conversion of carotene should be largely obtained by indirect means. Two main lines of investigation have been taken up. In the first place specimens of liver, butter, *etc.* obtained from different cows have been examined by the colorimetric method for carotene and vitamin A. In these experiments special attention has been directed to a comparison of specimens obtained from Jersey cows with those from other breeds, since the Jersey cow is reputed to show unusually deep pigmentation in its body- and milk-fat. Secondly an experiment has been carried out on the effects of feeding carotene, as carrots or red palm oil, on the degree of pigmentation and vitamin A content of butter-fat obtained from a cow at the end of the winter stall-feeding period.

The estimation of carotene and vitamin A.

Ox-liver. Specimens of liver from cows of known breed and history were kindly provided by Dr F. Carr, of British Drug Houses, Ltd. The extraction of the oil and colorimetric examination were carried out in the manner previously described [Moore, 1930; Dann and Moore, 1931]. The results obtained are shown in Table I. Vitamin A, as characterised both by a low yellow/blue ratio and by absorption at $m\mu$ 620 in the $SbCl_3$ test, was demonstrated in all cases, the amounts present varying considerably between different animals. The extracts were all somewhat yellow in colour, but the amount of carotene present, even in the most deeply pigmented samples, could have represented only a small fraction of the total chromogenic value.

Table I.

Sex and Age	Breed	Feeding	Yellow units per g. liver	Blue units per g. liver
Ox, 3 yrs.	Hereford	Grass	2	25
Ox, 3 yrs.	Black Angus	Grass	12	300
Ox, 4 yrs.	Devon	Grass	2	30
Bull, 7 yrs.	Jersey	Store	6	230
Cow, 7 yrs.	Jersey	Grass	18	30
Cow	Jersey	Unknown	9	350

Butter-fat. In view of the occasional commercial practice of increasing the yellow shade of butter-fat by the use of foreign colouring matter, it was essential that butter-fat of known history should be used. At the suggestion of Mr J. S. Penicud, milk recorder for the Cambridge district, samples of milk were collected from cows of the University Shorthorn herd through the

courtesy of Mr W. Mansfield, and from cows of Lady Walston's pedigree Jersey herd through the courtesy of Mr H. B. Hart. Collections were made on the same date at a time when both herds had been at grass for many weeks. On superficial examination it was obvious that the samples from the Jersey cows were in each case of a much more pronounced yellow shade than those given by the Shorthorns. At first an attempt was made to examine the samples individually, but the technique necessary to ensure the complete extraction of the fat was found to be too laborious to permit of this course. The samples from each herd were therefore combined, the cream was removed by skimming and converted into butter in a shaking machine. The butter-fats, after rendering, were examined colorimetrically both before and after saponification. The results obtained are shown in Table II.

Table II.

	Rendered fat		Unsaponifiable matter		
	Yellow units per g.	Blue units per g.	% unsaponifiable matter	Blue units calculated per g. original fat	Position of absorption bands in $SbCl_3$ reaction
Jersey cows	25	7·5	0·3	20	Dense vitamin A band 620 $m\mu$, hazier band 575 $m\mu$
Shorthorn cows	11	5·0	0·26	25	Vitamin A band present

The presence of vitamin A in both specimens was made apparent by the appearance of the 620 $m\mu$ band in the $SbCl_3$ test given by the unsaponifiable matter, although with the Jersey butter a fainter band at $m\mu$ 575 was also observed[1]. The yellow/blue ratios were consistent with this finding, indicating, on the unproven assumption that the yellow pigmentation was due entirely to unchanged carotene, that about 10 % and 5 % of the total blue value was attributable to the pigment in the Jersey and Shorthorn butter respectively. The decidedly greater yellow value given by the Jersey sample was in good accordance with the deeper colour of the original milk.

It will be noticed that the blue value calculated per unit of original fat was much increased by the process of saponification. The author's attention was directed to this point by Dr H. N. Green, who had previously observed the same phenomenon. The increase would seem to be caused by the removal of substances which interfere with the $SbCl_3$ reaction, similar increases being usually observed on saponifying cod-liver oils.

Blood-fat and body-fat. A few experiments were carried out upon specimens of body-fat and blood-fat obtained from animals of unknown history. In all cases only small amounts of chromogenic material were present, suggesting that in the cow, just as in the rat, the main reserves of vitamin A are concen-

[1] Heilbron and Morton described the position of the second band shown in New Zealand butter-fat (derived mainly from Jersey cows) as 592 $m\mu$, characteristic of carotene. The band at 575 $m\mu$ observed in the present experiments on the other hand, was in the same position as the band later detected by these workers in certain cod-liver oils.

trated in the liver. In general the colorations obtainable in the $SbCl_3$ test were too transient to permit of spectroscopic examination or even accurate matching in the tintometer. From a rough comparison of the yellow and blue values, however, it was obvious that the relative proportion of yellow pigment present was always much greater than in liver-fat.

Corpus luteum. A single specimen of dried cow's corpus luteum of unknown source was kindly made available by Mr C. W. Bellerby. The material was extracted and examined in the usual way. A yellow/blue ratio of 4 was given, while the main absorption band in the $SbCl_3$ test was at $m\mu$ 590, the position characteristic of carotene. While it would be unjustifiable on the evidence available to exclude the possibility that traces of vitamin A were present, it is obvious that the main fraction of the chromogenic value was in this case represented by a carotenoid.

Weight, powdered corpus luteum g.	Weight fat mg.	Total yellow units	Total blue units	$SbCl_3$ absorption band $m\mu$
1·9	80	600	150	590

The effect of carotene-feeding upon the carotene and vitamin A contents of butter-fat

In this experiment cows belonging to the National Institute for Research in Dairying were used, through the kindness of Mr James Mackintosh, who arranged for the feeding of the animals and the dispatch of samples of butter to Cambridge. The work was so timed that the feeding of the supplements containing carotene commenced in the early spring at a point when the carotene and vitamin A contents of the milk would have been expected to have stood at their lowest levels, as the result of maintenance through the winter months upon a diet relatively low in carotene. This diet consisted of two parts, (1) a "basal ration" of mangels 25 lbs., potatoes 5 lbs., oatstraw chaff 4 lbs., treacle 1 lb., hay 3–4 lbs., oats 2 lbs.; (2) an allowance of a mixture of oats 3 parts, dried grains 1 part, rice meal 2 parts, maize gluten feed 2 parts, decorticated ground-nut cake 1 part, soya meal 1 part, adjusted to the milk yield of the individual cow. Although no actual examination of these food materials was carried out several of the components, notably the mangels, maize gluten feed and soya meal may be suspected to have contained small amounts of carotenoids. At the same time the pale colour of the butter-fats at the commencement of the experiment made it plain that the carotene content of the diet as a whole was inadequate to permit pigmentation at the normal summer level.

At suitable intervals milk was collected from each cow and samples of butter were prepared, this part of the work being supervised by Miss Dearden. For this purpose the milk from two or three days had to be used, the intervals of collection have therefore usually been calculated from the first day on which samples were taken. After three batches of butter had been examined, in order to determine the preliminary levels of the vitamin A and carotene, the cows

were treated individually; (*a*) Winnie 3rd served as control receiving no supplement, (*b*) Campion 2nd received 30 lbs. of carrots daily in place of mangels, (*c*) Woodruff 2nd received 8 oz. red palm oil daily. The butter samples were placed in cold storage when received, and were examined later as opportunity arose.

Table III. *Yellow and blue values given by rendered butter-fats.*

	Winnie 3rd		Campion 2nd		Woodruff 2nd	
Sample	Yellow units per g.	Blue units per g.	Yellow units per g.	Blue units per g.	Yellow units per g.	Blue units per g.
0	10	12·5	4	7·5	8	7·5
6	7	12·5	3·5	5	9	7·5
13	4	10	2·5	5	6·5	7·5
	Negative control		Carrots 30 lbs. daily		Red palm oil 8 oz. daily	
20	3	10	2·5	10	4	7·5
28	3	10	6	10	5·5	7·5
30	3	10	7	12·5	6	8·5
36	2	7·5	7	12·5	5	8·5
43	7	10	9	12·5	8	7·5

The results obtained are given in Table III.

(*a*) The samples from the control animal, Winnie 3rd, showed a gradual decrease in yellow value, with the exception of the last sample taken, which, for reasons quite obscure, showed a partial recovery towards the original value. This decline in yellow coloration could easily be appreciated by the naked eye in the butter even before rendering, the four samples preceding the final being remarkably faintly coloured. The blue values given by the rendered fat showed a much less marked decline.

(*b*) The preliminary samples of butter-fat from Campion 2nd decreased both in yellow value and blue value. On feeding carrots, however, increases in both these values to levels considerably above the initial values were observed. Although eventually both values were increased in about the same proportion it is noteworthy that the increase in blue value was shown in sample 4, and thus definitely preceded the increase in yellow value, which was first shown in sample 5.

The significance of these results may be best appreciated by reference to Fig. 1. The broken curve has been drawn to represent the fraction of the blue values theoretically attributable to carotene, being deduced from the yellow curve on the assumption that carotene gives a yellow/blue ratio of about 11. It will readily be seen that the increase in blue value as the result of carrot feeding was much too great to be related to the increase in unchanged carotene. It is therefore obvious that the feeding of carotene led to the appearance of vitamin A in the butter-fat. Confirmation for this view was afforded by the predominance of the 620 $m\mu$ absorption band in the $SbCl_3$ reaction applied to unsaponifiable matter obtained from the various samples.

(*c*) The samples given by Woodruff showed declining yellow values during

the preliminary period. The feeding of red palm oil seemed to result in a restoration of the yellow colour towards its original value, although the results were irregular. The blue value remained practically constant throughout the whole experiment. The supplement was therefore less marked in its effect than the carrots, either on account of the relatively small amount given or through poor absorption.

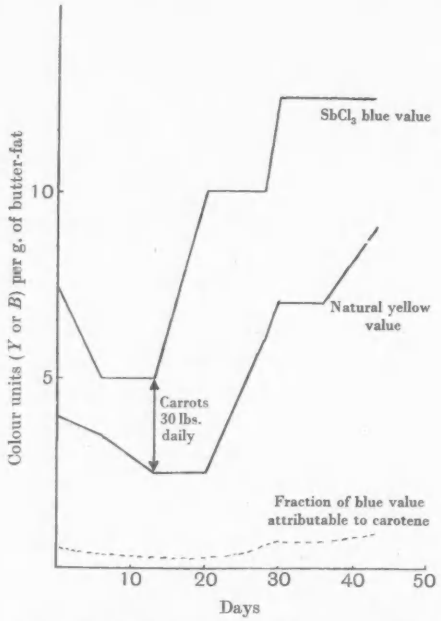

Fig. 1. Campion 2nd. The effect of carrot-feeding on the vitamin A and carotene content of the butter-fat.

Discussion.

A general consideration of the above evidence supports the view that the cow resembles the rat in its ability to convert carotene into vitamin A. Since the cow is exclusively herbivorous under natural conditions the invariable presence of vitamin A in the liver oil in itself would place this conclusion almost beyond doubt. At the same time the presence of carotene in the milk-fat and body-fat would seem to result from some genuine modification in its metabolism, and not merely from the large amounts which are normally included in the diet. Thus, in contrast with the cow, the sheep maintains colourless body-fat under identical conditions of nutrition, while the pig, in the experience of the writer, may consume large amounts of red palm oil without showing the least degree of pigmentation in its fat. The finding that the butter-fats of Jersey and Short-

horn cows show different degrees of pigmentation under similar conditions of nutrition indicates that the factors determining the presence of carotene in the fat may vary even between different breeds of the same animal.

Although in the liver of the cow vitamin A represents the main fraction of the chromogenic value it seems probable that in the rest of the body carotene is present in much higher proportion. The experiments of Euler, Euler and Hellström [1928] have indeed suggested that the blue colour given by ox blood-fat corresponds exactly with the carotene content. Of particular interest in this connection is the presence of carotene in the corpus luteum. Since Escher [1913] succeeded in isolating the crystalline pigment from mixed cow and sheep corpora lutea there can be no doubt as to its identity. The presence of such considerable amounts of carotene, apparently unaccompanied by vitamin A, must present an interesting problem in the mechanism of reproduction.

The economy of vitamin A and carotene in the cow during lactation.

The results obtained in the above experiments possess secondary interest in permitting a rough visualisation of the vitamin A economy of the cow. Considerations may be based on the following approximate data for an average cow.

(1) When having access to pasture, the cow may eat up to 40 kg. of grass daily. If the grass be taken to contain 10–100 mg. of carotene per kg. (fresh wt.) this intake must represent 0·4–4 g. of carotene, equivalent to 80,000–800,000 B.U. of the pigment, which should presumably be capable of yielding about 800,000–8,000,000 B.U. of vitamin A on complete conversion.

(2) The weight of the liver may be taken as roughly 5 kg. If the vitamin A content be taken as 300 B.U. per g., typical of the highest values obtained in the present experiments, the total reserve may therefore be computed as about 1,500,000 B.U.

(3) A cow may secrete about 1 kg. of milk-fat daily. This should involve the loss of 20,000 B.U., together with 10,000–20,000 Y.U., according to breed.

In the case of the cow at pasture, therefore, the daily output of vitamin A and carotene in the milk-fat is very small in comparison with the amount of carotene available in the diet. Storage of vitamin A in the liver takes place, while a large excess of pigment must undoubtedly be excreted unchanged. It is of interest to note that just as in the rat the vitamin A content of the body-fat remains within normal limits in the face of large dietary excess of carotene [Moore, 1931], so in the cow the vitamin A and carotene of the butter-fat are maintained at normal values under similar conditions. This point is most clearly illustrated by the experiment on Campion 2nd described above. During the period of experimental feeding the cow consumed about its own weight of carrots, an amount obviously sufficient, in the absence of selective absorption by the liver and rejection by the intestine, to render the body-fat and milk-fat deep orange in colour. Nevertheless the vitamin A and carotene content of the butter-fat did not rise above the normal summer level.

On the other hand if the cow is stall-fed upon a diet almost devoid of carotene the daily drain of vitamin A into the milk-fat can no longer be neglected. On the basis of the typical figures given above it is obvious that the reserves of vitamin A in the liver, assuming that they are available for transference to the milk-fat, would last for only 75 days. The reserves of carotene and vitamin A in the body-fat, from experiments on the rat, would be expected to represent a much smaller aggregate. Unless the liver has been unusually highly charged with vitamin A, therefore, the cow must be incapable of maintaining the concentration of the vitamin in its milk-fat at the normal level when exposed to long periods of carotene deficiency. In practical dairying a small allowance of carrots or other material rich in carotene should suffice to restore a positive balance.

Summary.

(1) Although the body-fat of the cow is normally characterised by the presence of small amounts of carotene, vitamin A as such is present in preponderating amounts both in the liver- and milk-fats. Since the cow is purely herbivorous this finding suggests that carotene undergoes the conversion to vitamin A previously demonstrated in the rat.

(2) This conclusion is supported by the finding that the feeding of carotene, in the form of carrots, to a cow at the end of the winter stall-feeding period resulted in an increase not only in the carotene content but also in the vitamin A content of the butter-fat.

(3) The degree of pigmentation of butter-fat, when adequate carotene is available in the diet, is determined by breed. Butter-fat derived from Jersey cows gave a yellow value twice that obtained with butter-fat derived from Shorthorn cows under similar nutritive conditions.

(4) When the cow is at pasture the output of carotene and vitamin A in the butter-fat is very small in comparison with the amount of carotene available in the diet. On the other hand during stall-feeding upon a diet almost devoid of carotene the reserves of vitamin A available in the liver and elsewhere may be insufficient to meet the demands of prolonged lactation. In practical dairying a small allowance of carrots, or other material rich in carotene, should suffice to restore a positive balance.

My thanks are due to Dr L. J. Harris for his valuable criticism. I must also express my indebtedness to Mr James Mackintosh, of the National Institute for Research in Dairying, not only for having arranged the experiments on dairy cows but also for supplying much of the information used in writing the discussion.

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II. MILK PEROXIDASE.

ITS PREPARATION, PROPERTIES, AND ACTION WITH H_2O_2 ON METABOLITES.

WITH A METHOD FOR DETERMINING SMALL AMOUNTS OF H_2O_2 IN COMPLEX MIXTURES.

By KENNETH ALLAN CALDWELL ELLIOTT[1].

From the Biochemical Laboratory, Cambridge.

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It is generally believed that peroxidases are almost universally present in the tissues of animals and widely distributed in plants and bacteria, but though there is a large literature concerning them, it has not yet been shown experimentally that they serve any physiological function. It is even doubted whether their specific oxidising substrate, hydrogen peroxide, ever occurs significantly in normal living matter, since no positive test in such material has been obtained. But it is certain that H_2O_2 is formed by certain oxidising systems separated from living matter; thus H_2O_2 has been shown to be formed in the aerobic oxidations promoted by xanthine oxidase, aldehyde oxidase, succinic oxidase, tyramine oxidase, and the catechol oxidase of potatoes, and also in the oxidation of cysteine and glutathione [Thurlow, 1925; Kodama, 1926; Hare, 1928; Onslow and Robinson, 1926], and the work of M'Leod and Gordon [1922] and of Avery and Neill [1924, 1925] makes it clear that hydrogen peroxide is produced in bacterial metabolism.

Thus it seems likely that H_2O_2 could be a normal tissue product. The difficulty in detecting it in normal tissues may be quite easily accounted for. Peroxidase acts at extraordinarily low concentrations of H_2O_2, and if reducing substrates are present, the H_2O_2 would be removed by this mechanism as soon as it is formed; also reduced glutathione present in the tissues would use up H_2O_2, and Keilin [1930] has shown that reduced cytochrome-*c* is readily oxidised by H_2O_2. Finally any accumulation would be destroyed by catalase which appears to be present in all tissues.

Dakin [1905–1909] showed that many biological substances could be oxidised by H_2O_2, in some cases with iron catalysis, to give products similar to those met with in studies of intermediary metabolism. This, and the fact that H_2O_2 formed by aerobic oxidising systems could be made to perform secondary oxidations [Thurlow, 1925; Harrison and Thurlow, 1926; Szent-

[1] Beit Memorial Research Fellow.

Györgyi, 1928; Wieland and Rosenfelt, 1930] suggested that hydrogen peroxide might be an important biological oxidising agent. In Thurlow and Harrison's work haemoglobin was oxidised in the secondary stage to methaemoglobin, and, with iron as catalyst, lactate and β-hydroxybutyrate were oxidised. But the only oxidations they observed to be catalysed by peroxidase were those of nitrite and an ether-soluble substance, presumed to be a fat, present in the milk from which the enzyme itself was prepared, and only present in summer. Szent-Györgyi [1928] demonstrated the oxidation by H_2O_2 and peroxidase of phenolic substances, such as adrenaline, which in turn oxidised the hexuronic acid found by him in plants and the adrenal cortex of animals, and he claimed to have shown a connection between this system and the glutathione sulphydryl-disulphide system.

The presence of peroxidase is detected by colour reactions with H_2O_2 and a large variety of organic compounds, but the only substances of biological interest known to be oxidised by this enzyme and H_2O_2 are adrenaline (Szent-Györgyi) and the rather doubtful ether-soluble substance of Harrison and Thurlow mentioned above. Thus, though peroxidase is so widely distributed, with H_2O_2 quite possibly a regular metabolite, there is no direct evidence for its having any physiological function except for the complicated, and limited, scheme of Szent-Györgyi.

It was felt that more knowledge of the powers of peroxidase with H_2O_2 as an oxidising agent for substances of metabolic interest might throw light on its possibilities as a biological mechanism. The main object of the work to be described has therefore been to see what possible metabolites can be oxidised by H_2O_2 and an animal peroxidase under more or less physiological conditions.

Batelli and Stern [1908] claimed to have shown the oxidation of formate, with evolution of CO_2, by H_2O_2 with various tissues, especially liver, the catalyst responsible being thermolabile. However, their methods were crude; H_2O_2 was used in concentrations far greater than could occur in normal tissue, and probably sufficient to destroy any true peroxidase; the formate concentration was also very high, and the p_H was unknown but was certainly kept very low in order to diminish destruction of the hydrogen peroxide by catalase. Using a refined method with a more purified enzyme preparation in proper conditions their results have not been confirmed by me.

Preparation of the enzyme.

The peroxidase of milk was chosen as being a true peroxidase of animal origin, easily prepared as a soluble solid free from other enzymes, especially catalase.

Thurlow [1925] used a preparation of this enzyme obtained by mixing milk with an equal volume of saturated ammonium sulphate solution, filtering the precipitated caseinogen and fat, and then saturating the filtrate with ammonium sulphate. The lactalbuminous precipitate thus obtained gave

fairly active solutions and was used in her experiments. Her method is here modified in view of the following observations.

If, instead of a fresh ammonium sulphate solution, a filtrate (containing lactose *etc.*) from a previous preparation was used for the half-saturation of milk, the peroxidase activity was deposited entirely on the caseinogen precipitate leaving the filtrate inactive. Similar results are obtained when using a fresh ammonium sulphate solution if a few extra crystals of the salt are added as well. Thus a slight excess of crystalloidal matter is sufficient to determine the loss of all activity in the filtrate, and by the Thurlow method quite a considerable amount of the enzyme is lost on the caseinogen. (The enzyme can be washed off the caseinogen precipitate again by a one-third saturated solution of ammonium sulphate.) For the purpose of this work, however, it was not permissible to add to the milk only the minimum amount of salt required to precipitate the caseinogen, as it was very necessary to deposit the catalase and this is only accomplished on approaching half-saturation [mentioned by Raudnitz, 1901].

If solid ammonium sulphate is added gradually to the active filtrate free from caseinogen and fat, it is found that the enzyme is deposited entirely on the first protein precipitate formed so that activity disappears quite sharply from the liquid phase. Saturation with the salt is therefore not necessary, as it simply brings down more inactive protein.

Below is described a typical example of the new preparation, which has the following advantages. By adding solid ammonium sulphate instead of a saturated solution, the volumes are kept smaller and a considerable economy of the salt is effected. If the correct amount of ammonium sulphate is used, the resulting preparation contains a negligible amount of catalase. By adding only just sufficient salt in the final precipitation of the enzyme, the amount of protein accompanying the enzyme is diminished to about one-third, and far less ammonium sulphate is present in the preparation than when the liquid has been saturated.

To 8 litres of fresh milk 2000 g. of solid ammonium sulphate were added. (The amount of salt to be added was always determined by adding various amounts of the salt to 50 cc. samples of the milk to be used, and, by qualitative tests[1], finding the greatest amount of the salt that could be added without appreciably diminishing the peroxidase activity of the filtrate. As mentioned above, if too much salt is added the peroxidase is lost on the caseinogen, if too little, then the preparation will be contaminated with catalase.) On filtering through large fluted filter-papers, 7350 cc. of a clear filtrate were obtained. To the filtrate 1240 g. of ammonium sulphate were added. (By filtering small samples during the addition and testing the activity of the filtrates this amount was found to be sufficient to precipitate the enzyme completely.) On filtering, and drying the precipitate between filter-papers and in a vacuum desiccator, 20·3 g. of the active solid were obtained.

[1] In all qualitative tests for peroxidase it was found best to add the H_2O_2 in 0·02 % solution.

The inactive filtrate was then saturated with ammonium sulphate by the addition of a further 1600 g.; the dried lactalbumin precipitate of 43 g. was completely inactive and was used for certain control experiments to be described later.

The milk had a "purpurogallin number" of 0·0225 and therefore contained 180 units of peroxidase in the 8 litres, and the filtrate free from caseinogen and fat had a purpurogallin number of 0·010 and therefore contained 73·5 units. (If it were not necessary to get rid of small amounts of catalase, a much higher yield could be obtained by adding less salt.) The solid preparation had a purpurogallin number of 2·21, and contained therefore in the 20·3 g., 44·9 units corresponding to a yield of 25 % of the activity of the milk.

Preparations of more than twice the strength of the above have been obtained by the same method.

The estimations of peroxidase activity were made by the method of Willstätter and Stoll [1918]. In estimating the activity of milk 0·9 cc. of the milk was required, which caused a thick emulsion to be formed in the ether extraction; this emulsion was best broken by small additions of alcohol. With the other enzyme preparations the emulsion could usually be broken by the addition of sodium chloride.

Properties of the enzyme.

A solid preparation when kept in the ice-chest loses its activity very slowly; for example, in 6½ months at 0–5° the activity of one sample dropped from a purpurogallin number of 3·6 to 2·4.

The enzyme is active over a wide range of hydrogen ion concentration. In the alkaline range from p_H 8 to nearly 10 the activity is diminished but recovers immediately on neutralising; there is still a trace of activity at p_H 10 though the enzyme is almost completely and irreversibly destroyed. In the acid range, between p_H 4·2 and 3·8 a certain amount of precipitate is formed and the activity is diminished; between p_H 3·6 and 3·2 the precipitate redissolves and all activity disappears. On bringing these acid solutions to about p_H 7 and allowing to stand overnight, the activity is regenerated to some extent; this interesting observation is worth further study.

It will be noted that the peroxidase from milk is more sensitive to acidity than that from horse-radish. In consequence it is impossible to use the malachite green method of Willstätter and Weber [1926] for estimating activity, as this method necessitates working at p_H 3·5 to keep leuco-malachite green in solution, and at this acidity milk peroxidase is quite inactive.

Up to p_H 7 this peroxidase oxidises benzidine to give a blue colour which usually changes to brown. In more alkaline solutions the benzidine is oxidised directly to the brown substance. *p*-Phenylenediamine gives a blue colour throughout the range of activity of the enzyme.

In the experiments on the preparation of the enzyme, it was noticed that whenever a clear active solution was obtained a slight but distinct brown colour was present, while inactive solutions were considerably less coloured; a concentrated solution of the solid preparation was strongly coloured. The

solid on reduction and treatment with pyridine gave a haemochromogen spectrum similar to, but not the same as, that given by a horse-radish preparation when treated in the same way. The indications are that some coloured haematin body is connected with the enzymic activity, and this question is being studied further.

Effects of sulphydryl compounds on tests for peroxidase.

The inhibitory effect of very small amounts of H_2S on the activity of peroxidases is well known. It was found that various other sulphydryl compounds interfere with the test when using benzidine, tincture of guaiacum, or *p*-phenylenediamine as substrate for the peroxidase and H_2O_2. Solutions of egg- or milk-albumin, which have been denatured by boiling in alkali and then neutralising, give a nitroprusside test for —SH, and when added to a peroxidase solution prevent the blue colour forming with H_2O_2 and benzidine or guaiacum; the formation of colour with *p*-phenylenediamine is noticeably slowed. Neutral solutions of cysteine and reduced glutathione have the same effect. In boiled milk the enzyme is destroyed and a slight positive nitroprusside test is given; adding boiled milk to fresh milk will cause some interference with the peroxidase test using benzidine, guaiacum or *p*-phenylenediamine.

However, the action of these sulphydryl substances is not apparently an inhibition of the enzyme, as is that of H_2S, but is due to their reducing action on the coloured products of the oxidations. Whereas 0·1 cc. of *M*/100 H_2S solution will inactivate 5 cc. of a solution of the enzyme, only some delay in the colour formation from *p*-phenylenediamine is caused by 2 cc. of *M*/100 cysteine solution. Guaiacum is more sensitive, 0·4 cc. causing a delay; and benzidine is very sensitive, 0·2 cc. of the cysteine solution completely preventing the formation of the blue colour. It is found that addition of —SH compounds to benzidine blue immediately decolorises it; guaiacum blue is fairly rapidly decolorised. The effect on *p*-phenylenediamine blue is scarcely noticeable for some hours.

The reaction with pyrogallol is not affected by cysteine or denatured protein, and it was found that purpurogallin is not decolorised by —SH compounds.

Estimation of hydrogen peroxide.

As the chief method of attacking the problem was to see whether hydrogen peroxide was used up in the presence of peroxidase and the substances in question, it was necessary to devise a method for estimating small quantities of H_2O_2 in the presence of proteins and other substances which would interfere with the usual methods. It was decided to make use of the catalytic decomposition of H_2O_2 by manganese dioxide, and to measure the oxygen evolved.

Two 100 cc. Erlenmeyer flasks were fitted with rubber stoppers through which passed glass tubes connected by pressure tubing with a Haldane

evolution manometer [Haldane, 1920] to measure 1 or 2 cc. of gas. One of the glass tubes was so made that a short test-tube could be suspended from it by means of a platinum hook. The suspended tube contained about 0·2 g. of manganese dioxide powder and the flask in which it was hung contained the liquid the H_2O_2 content of which was to be measured. The other flask contained an equal volume of water and was connected up as temperature compensator. The two flasks were clamped close together in a tank so that they could be steadily shaken while up to their necks in water at room temperature. They were shaken gently for 20 minutes or more until equilibrium was reached on the manometer. The manometer was then set, and the suspended tube was jerked into the liquid and that flask vigorously shaken by hand for about 5 minutes. The two flasks were then again shaken steadily until equilibrium was reached, and the volume of gas evolved was read off on the manometer.

Table I. *Gas evolved on shaking various solutions with MnO_2.*

In all cases the total volume was made up to 35 cc. 10 cc. of the hydrogen peroxide solution (0·02 %) give theoretically 0·66 cc. of O_2 at 15° on catalytic decomposition.

H_2O_2 solution cc.	20 % H_2SO_4 cc.	*M*/15 phosphate buffer cc.	Other solutes	Gas evolved cc.
10	—	—	—	0·79 0·81 0·72
10	—	20	—	1·16 1·12 1·20
10	—	8	—	1·16
10	3	—	—	1·32
10	—	—	1 g. lactalbumin	1·16
10	3	20	—	1·30
10	—	20	1 g. lactalbumin	1·23 1·09
10	3	20	1 g. lactalbumin	1·30
10	—	—	0·4 g. peroxidase preparation	1·02
10	—	20	0·4 g. peroxidase preparation	1·16
10	3	20	0·4 g. peroxidase preparation	1·30
10	—	—	1 g. egg-albumin	0·83
10	—	—	0·5 g. gelatin	0·77
10	—	—	Albulactin	0·94 0·95 0·93
10	—	—	0·5 g. Na_2SO_4	0·83
10	—	—	1 g. $(NH_4)_2SO_4$	0·90
5	—	—	—	0·43
2·5	—	—	—	0·18
5	3	—	—	0·70
5	—	20	—	0·63
2·5	—	20	—	0·33
5	3	20	1 g. lactalbumin	0·68
5	—	—	1 g. lactalbumin	0·62

The reaction of MnO_2 with hydrogen peroxide is not always a simple catalytic decomposition of H_2O_2, and it was found that the presence of various substances affected the reaction, all causing evolution of O_2 to be in excess of the amount required by the equation, $H_2O_2 = H_2O + \frac{1}{2}O_2$. For instance,

when the O_2 evolved according to this equation should be 0·7 cc., the presence of phosphate caused the production of 1·2 cc. of gas, though variations in the concentration of phosphate did not affect this amount. Lactalbumin had a similar effect, and other proteins and salts also caused increased evolutions of gas. In the presence of sulphuric acid 1·3 cc. were evolved, and this amount was not affected by other substances in solution as well. These results are illustrated in Table I. It is probable that these substances cause the reaction $MnO_2 + H_2O_2 = MnO + H_2O + O_2$ to take place to some extent; with sulphuric acid manganous sulphate would be formed.

In all cases it appears that the evolution of O_2 remains proportional to the amount of H_2O_2 initially present, when solutions containing the same solutes but varying amounts of H_2O_2 are estimated. Thus the method is suitable, and very convenient, for estimating roughly and comparatively very small amounts of H_2O_2 (in these experiments, a maximum of 2 mg. H_2O_2 in 35 cc.) in a mixed solution, provided the other substances in solution are the same in each case. Rough absolute values for H_2O_2 concentrations could be obtained by standardising against known H_2O_2 solutions containing the same solutes. It is recommended that sulphuric acid should be added to the liquid before estimating. As is seen in Table I, the greatest yield of gas is then obtained and it appears not to be affected by other solutes.

When estimating H_2O_2 in an acid solution it is very necessary to use MnO_2 that has been previously thoroughly washed with acid to remove traces of carbonate that are often present.

Oxidations by peroxidase and H_2O_2.

The method used consisted simply in slowly adding dilute hydrogen peroxide to a solution containing both peroxidase and the substance under experiment. After a suitable time the H_2O_2 was estimated by the manganese dioxide method. If at the end of the experiment there was as much H_2O_2 remaining as in a control without the substance, it could be concluded that the substance was not oxidised. If, however, there was a disappearance of peroxide (and a further control without the enzyme but with the substance showed no uncatalysed usage of H_2O_2) it was concluded that the enzyme *plus* the substance under experiment were using up H_2O_2, *i.e.* oxidation was occurring.

From the work of Bach [1904], Willstätter and Weber [1926], and Mann [1931] on the peroxidase of horse-radish, it is known that excess of H_2O_2 inhibits the activity of the enzyme and, in larger amounts, destroys it. It was therefore necessary to work with very low concentrations of H_2O_2. In the method used the maximum concentration of H_2O_2 was 2 mg. in 35 cc. ($1{\cdot}4 \times 10^{-3}$ *M*), and if the peroxide was used up at all rapidly its concentration would not exceed 0·1 mg. in 35 cc. (or 7×10^{-5} *M*). With such concentrations Willstätter and Weber, and Mann, with the vegetable peroxidase

and pyrogallol, leuco-malachite green, or guaiacol, found their enzyme quite active. Destruction of enzyme was found not to be significant. Mann showed, with the vegetable peroxidase, that very high concentration of the reducing substrate had an inhibitory effect. The effect was not very large, but nevertheless the concentrations of substances to be tested were always made small, though more than sufficient to use up all the H_2O_2 if they were being oxidised by it.

Nitrite.

Before making experiments on substances of more biological interest, it was decided to see whether the method to be used would give clear-cut results with nitrite, since this substance is known to be oxidised by peroxidase and H_2O_2 [Haas and Lee, 1924; Thurlow, 1925].

The following solutions were used.

A 10 % solution of a Thurlow[1] peroxidase preparation in water or phosphate buffer, adjusted roughly with dilute sodium hydroxide to p_H 7.

A 0·02 % solution of hydrogen peroxide. (Diluted from Merck's "perhydrol.")

A solution of sodium nitrite containing 1 mg. $NaNO_2$ per cc.

The following seven experiments were made in 100 cc. Erlenmeyer flasks, A to G.

Flask A contained 20 cc. peroxidase solution and 8 cc. nitrite solution. While the flask was kept at 38–40° in a bath and gently shaken, 10 cc. of the hydrogen peroxide solution were slowly added from a burette fixed in the stopper of the flask during 45 minutes, and the flask was kept warm for a further 45 minutes. It was then cooled and connected with the estimation apparatus and the hydrogen peroxide remaining estimated. No O_2 was evolved showing that the H_2O_2 had all been used up, the nitrite added having been more than the equivalent of the hydrogen peroxide.

The procedure was the same in the remaining six experiments, the contents of the flasks varying as follows:

B. Only 3 cc. nitrite solution, *plus* 5 cc. water.

C. No nitrite, 8 cc. water.

D. Same as B, but 10 cc. of water were added instead of the H_2O_2 solution.

E. Same as B, but the peroxidase solution was first inactivated by heating to 85–90° for 7 minutes.

F. Peroxidase solution of only half the usual strength was used and only 1 hour instead of 1½ hours was allowed.

G. The same enzyme preparation was used after it had lost most of its activity on standing 6 weeks at room temperature. 2·5 cc. nitrite solution.

The results of these experiments are summarised in Table II. A further control experiment showed that H_2O_2 and manganese dioxide did not oxidise

[1] This experiment was done before the improved preparation was devised. Similar results were later obtained with much more dilute solutions of the stronger preparations.

the nitrite. Similar experiments using half the strength of H_2O_2 and less nitrite gave similar results.

In A, B and F, when nitrite was being oxidised a yellow colour developed; in none of the other experiments did it appear, and it is probable that it is due to a xanthoproteic reaction given by the nitrate formed, as addition of ammonia was found to deepen the colour to orange.

Table II. *Oxidation of nitrite by H_2O_2 and peroxidase.*

Exp. No.	Characteristic	H_2O_2 unused cc. O_2 evolved by MnO_2	Griess-Ilosvay test at end for nitrite	Xantho-proteic (?) colour formed during exp.
A	Excess nitrite	0·02	+ + + +	+ +
B	Low nitrite	0·34	–	+
C	No nitrite	1·14	–	–
D	No H_2O_2	0·0	+ + + +	–
E	Heat-inactivated enzyme	0·96	+ + + +	–
F	Half amount of enzyme. Less time. (Low nitrite)	0·37	+	+
G	Age-inactivated enzyme	0·75	+ + +	–

Possible metabolites.

Having shown that the method gives clear results with nitrite, various substances of metabolic interest were tried. In each series of experiments, especially when new enzyme preparations were being used, an experiment with nitrite was also done in order to make sure that the conditions were correct.

In the following experiment formate, glycine, glucose and oleic acid were tried.

Six 100 cc. Erlenmeyer flasks, each fitted with a 10 cc. burette fixed in the stopper, were set up. Each contained 20 cc. of an aqueous 2 % solution of a peroxidase preparation of purpurogallin number 4·3, roughly adjusted with $N/10$ soda to p_H 7. In addition 5 cc. of the following solutions were added to the respective flasks: 5 mg./cc. sodium formate, 5 mg./cc. glycine, 5 mg./cc. glucose, 10 mg./cc. oleic emulsion, 3 mg./cc. sodium nitrite, and distilled water. The "oleic emulsion" was prepared by dissolving oleic acid in the equivalent amount of NaOH solution and then adding acid till the reaction was about p_H 7·8. The p_H of the peroxidase solution was not appreciably affected by 5 cc. of this emulsion.

During 45 minutes' gentle shaking in a bath at 38–40°, 10 cc. of 0·02 % hydrogen peroxide were added in 0·5 cc. lots every $2\frac{1}{2}$ minutes from the burette attached to each flask. The flasks were allowed to stand another 20 minutes in the bath and then a 1 cc. sample was withdrawn from each flask, before adding 3 cc. of 20 % sulphuric acid. The addition of acid stabilised the remaining hydrogen peroxide which was estimated by the manganese dioxide method at room temperature as soon as convenient.

To the 1 cc. samples a couple of drops of *p*-phenylenediamine solution

were added. If colour developed it showed that both H_2O_2 and peroxidase activity were still present. If no colour formed, a little peroxide solution was added as well to see whether the enzyme was still active.

A seventh flask contained the peroxidase solution, the whole 10 cc. H_2O_2 solution was run in at once and the acid was added immediately without the 65 minutes in the water-bath.

The results of these experiments are summarised in Table III.

Table III.

		Formate	Glycine	Glucose	Oleic acid	Nitrite	Water	Acid added before exp.	Theory for 10 cc. H_2O_2 solution
1 cc. sample	Colour formed on addition of *p*-phenylene-diamine	+	+ +	+	+ +	−	+		
	plus H_2O_2	+	+ +	+	+ +	+ +	+		
H_2O_2 remaining at end. cc. O_2 evolved by MnO_2		0·68	0·57	0·58	0·57	0·00	0·62	1·29	0·68

In the control with immediate addition of acid it is seen that 1·29 cc. of gas were evolved instead of the theoretical 0·68 cc. This has been remarked on above. The difference between this control and the experimental flask containing only water and no reducing substrate shows the amount (1·29 − 0·62 = 0·67 cc.) of H_2O_2 which has been destroyed during the experiment by traces of catalase.

In the presence of excess nitrite we see again that the H_2O_2 has been completely used up, but with none of the other substances tried has there been any diminution of the H_2O_2 below that found in the water control. The qualitative tests also show that the enzyme is active at the end of the experiment in all cases, and that H_2O_2 has disappeared in the presence of nitrite but not in the presence of the other substances.

It can therefore be concluded that formate, glycine, glucose and oleic acid are not oxidised by peroxidase and H_2O_2, at least in conditions in which nitrite is quantitatively oxidised, and such substances as benzidine, pyrogallol and *p*-phenylenediamine are readily oxidised.

The result with formate is in contradiction to the finding of Batelli and Stern [1908] with liver and other tissues. They measured the extra CO_2 produced by formate oxidation. The above experiment with formate was repeated using a partially evacuated flask so that the peroxide solution could be added, and acid run in at the end of the experiment, without letting gas escape. After adding acid, samples of the gas in the flask were withdrawn into a Haldane gas analysis apparatus and the CO_2 estimated. No more CO_2 was found in the gas after an experiment with formate than in a control without.

In Tables IV–VII are given results of experiments carried out exactly similarly to those summarised in Table III.

For the results shown in Table IV the solutions used were: peroxidase preparation same as for Table III but the solution here used contained only 1·6 % of the solid, *p*-phenylenediamine 10 mg./cc., tryptophan 10 mg./cc. adjusted to p_H 7, sodium nitrite 3 mg./cc., stearic emulsion containing 5 mg./cc. stearic acid, prepared similarly to the oleic emulsion. 5 cc. of each were used, the tyrosine was added as 100 mg. finely ground crystals with 5 cc. water, the reaction fluid thus becoming saturated with this slightly soluble substance.

Table IV.

		p-Phenylene-diamine	Trypto-phan	Stearic acid	Tyrosine	Nitrite	Water
Sample	Colour with *p*-phenylene-diamine	.	+ +	+	−	−	+
	plus H_2O_2	.	+ +	+	+ +	+ +	+
Colour formed during experiment		Dark	Yellow-brown	.	Yellow-brown	Yellow	.
H_2O_2 remaining at end. cc. O_2 evolved by MnO_2		0·0	0·15	0·70	0·00	0·00	0·70

Here the H_2O_2 has been used up by nitrite, *p*-phenylenediamine, tryptophan and tyrosine, the last three giving coloured products.

The results in Table V were obtained with a 1·6 % solution of a new enzyme preparation of purpurogallin number 4·9. Only 50 mg. of powdered tyrosine were used. The tryptophan, phenylalanine and glycerol solutions each contained 10 mg. per cc.

Table V.

		Nitrite	Water	Tyrosine	Trypto-phan	Phenyl-alanine	Glycerol
Sample	Colour with *p*-phenylene-diamine	−	+ +	−	−	+ + +	+
	plus H_2O_2	+ + + +	+ +	+ + + +	+ + +	+ + +	+
Colour formed during experiment		Yellow	.	Brown	Brown	.	.
cc. O_2 evolved by MnO_2		0·00	0·60	0·00	0·04	0·53	0·43

This confirms the results with tyrosine and tryptophan; phenylalanine and glycerol gave negative results.

For the results in Table VI the peroxidase solution contained 1·2 % of the same preparation as in Table V. 5 cc. each of the following solutions were used: acetaldehyde 5 mg./cc., sodium acetate, histidine and phenylglyoxal each 10 mg./cc.

No colour was formed (except with nitrite) and acetaldehyde, acetate and histidine are evidently not oxidised. Phenylglyoxal has used up H_2O_2, but this is further discussed below.

Table VI.

		Nitrite	Water	Acet-aldehyde	Acetate	Histidine	Phenyl-glyoxal
Sample	Colour with *p*-phenylene-diamine	–	++	+++	++	++++	Yellow
	plus H_2O_2	+++	++	+++	++	++++	Yellow
cc. O_2 evolved by MnO_2		0·00	0·50	0·62	0·50	0·45	0·08

A 1·6 % solution of the same enzyme preparation as in Tables V and VI was used for the experiments of Table VII. 5 cc. of each of the following solutions were used in the respective experiments: ethyl alcohol 5 mg./cc., sodium lactate 10 mg./cc., dihydroxyacetone 10 mg./cc., an emulsion of triolein containing 10 mg./cc., and phenylglyoxal 10 mg./cc.

Table VII.

		Ethyl alcohol	Lactate	Dihydroxy-acetone	Triolein	Phenyl-glyoxal
Sample	Colour with *p*-phenylene-diamine	+	+	Yellow	+	Yellow
	plus H_2O_2	+	+	.	+	.
cc. O_2 evolved by MnO_2		0·53	0·49	0·26	0·43	0·0

No colour was formed in any of these experiments. Dihydroxyacetone and phenylglyoxal react with *p*-phenylenediamine giving a coloration which obscures the peroxidase reaction. Alcohol, lactate and triolein are apparently not oxidised. Dihydroxyacetone, as well as phenylglyoxal, uses up H_2O_2, but this is further discussed below.

Besides those substances appearing twice in the above Tables, the experiments with formate, glucose, glycine, phenylalanine, histidine and oleic acid were all repeated with different enzyme preparations with the same negative results.

A single experiment with β-hydroxybutyrate also gave a negative result. The experiments with phenylglyoxal and dihydroxyacetone were both done twice; they use up H_2O_2, but, as will be shown, this is not due to peroxidase activity.

Of all the substances tried above the only ones to take up H_2O_2 were tyrosine, tryptophan, phenylglyoxal and dihydroxyacetone; also *p*-phenylene-diamine which was tried as a further test of the method. It was necessary to see whether these oxidations were catalysed by peroxidase or whether the oxidation would take place in the absence of the enzyme. This possibility was controlled by making experiments in exactly the same way as before but without the enzyme; to make the conditions in the controls resemble those in the experiments as closely as possible the completely inactive lactalbumin preparation mentioned above was used in place of the enzyme preparation. Table VIII summarises the results of these controls, together with a test to

make sure that delay in estimating the H_2O_2 in acidified solutions does not appreciably affect the results. The lactalbumin used, unlike the enzyme preparation, contained no traces of catalase so that larger amounts of H_2O_2 are left at the end of the experiments.

Table VIII.

cc. O_2 liberated by MnO_2.

Water	Water (estimated after standing overnight)	Tryptophan	Tyrosine	Dihydroxyacetone	Phenylglyoxal	*p*-Phenylenediamine
1·34	1·27	1·33	1·33	0·24	0·04	0·28

It is seen that there is no trace of peroxide usage by tyrosine or tryptophan in the absence of peroxidase, nor was any colour formation whatever observed during the addition of H_2O_2. The H_2O_2 usage and colour formation with these substances in the presence of peroxidase must therefore have been catalysed by the enzyme.

p-Phenylenediamine, as would be expected, was oxidised and used up H_2O_2 to some extent without the enzyme. The enzyme of course accelerates the oxidation greatly. Dihydroxyacetone and phenylglyoxal both used up H_2O_2 to the same extent as in the presence of the enzyme; their oxidation by H_2O_2 is therefore not catalysed by peroxidase.

Discussion.

A representative selection of substances which are of interest in animal intermediary metabolism has thus been subjected to the influence of hydrogen peroxide and milk peroxidase, under conditions which are approximately optimal for the working of the enzyme and in which nitrite is quantitatively oxidised by the system.

With the following substances, probably concerned in fat, carbohydrate and protein metabolism respectively, no oxidation could be obtained:

Formate	Glucose	Glycine
Acetate	Glycerol	Phenylalanine
Oleate	Acetaldehyde	Histidine
Stearate	Lactate	
Triolein	Ethyl alcohol	
β-Hydroxybutyrate		

Oxidised by H_2O_2 without catalysis by the enzyme were:

Dihydroxyacetone Phenylglyoxal

Only two substances were found to be oxidised by H_2O_2 with peroxidase catalysis, namely,

Tryptophan Tyrosine

Thus the attempt to find any general activity of the system which might throw light on the physiological function of peroxidase has failed. It is possible that the enzyme from milk may differ fundamentally from peroxidases found in active tissues; but this seems unlikely since it is a very active enzyme and will oxidise all the substances which are used in the ordinary qualitative tests for other animal peroxidases, and in general seems likely to have the same properties as they have.

In view of the reported wide distribution of peroxidases it is difficult to dismiss them as serving no important physiological function, and it is not yet necessary to do so. The indophenol oxidase which is now very widely believed to be the central enzyme of tissue respiration, activates oxygen, but not directly to oxidise the various organic substrates, as it needs both an intermediate carrier, cytochrome, and the activation of the organic substrates by dehydrogenases [Keilin, 1929]. It is possible that peroxidase may be simply a counterpart to the indophenol oxidase, using H_2O_2 instead of O_2 as oxidising substrate, and needing the activation by other enzymes of most of its organic reducing substrates, and possibly the mediation of cytochrome as well. Cytochrome is oxidised by H_2O_2 alone [Keilin, 1930]; whether this oxidation is accelerated by peroxidase is not yet known. The system known as "succinic oxidase" has been shown [Fleisch, 1924; Keilin, 1929] to consist of the complete system, dehydrogenase + cytochrome + indophenol oxidase. It has been claimed [Thurlow, 1925] that this system with succinic acid and oxygen produces H_2O_2, and if this is so it is probable that H_2O_2 is formed in all oxidations in which indophenol oxidase is concerned. Hence the collaboration of peroxidase with indophenol oxidase may be advantageous to the efficient use of oxygen.

It is interesting to note that the two substances, tryptophan and tyrosine, found to be oxidised by peroxidase and hydrogen peroxide both give coloured products, as do all the non-biological organic substances which are oxidised by the system. It would however be premature to suggest pigment formation as a function of the enzyme.

A study of the powers of a vegetable peroxidase as a catalyst of oxidations by H_2O_2 is now in progress.

Summary.

1. A preparation of milk peroxidase by fractional precipitation with ammonium sulphate is described, whereby a crude but considerably concentrated preparation is obtained practically free from catalase.

2. The activity of the preparation degenerates very slowly in the refrigerator at 0 to 5°. The enzyme is active from about p_H 4 to nearly p_H 10. A colour appears to be associated with the enzyme, and haemochromogen bands can be obtained.

3. Compounds containing the —SH group, such as cysteine, glutathione, and denatured proteins, interfere with tests for peroxidase with benzidine,

guaiacum, and to some extent with *p*-phenylenediamine. This appears not to be an inhibition of the enzyme as with H_2S, but seems to be due to the reduction of the coloured reaction products by —SH compounds.

4. A method of estimating H_2O_2 in very small amounts (1 mg. in 35 cc.) by measuring the evolution of O_2 by manganese dioxide is described. The volume of gas obtained is affected by the presence of proteins, phosphate, acid, *etc.*, but in given conditions it is proportional to the H_2O_2 initially present.

5. Conditions were devised by which nitrite could be quantitatively oxidised by hydrogen peroxide with peroxidase, as shown by the usage of H_2O_2 and the disappearance of nitrite.

6. Under similar conditions tyrosine and tryptophan were found to be oxidised to coloured products.

7. Under the conditions in which nitrite is quantitatively oxidised, no oxidation by H_2O_2 and peroxidase of the following substances was obtained: formate, acetate, oleate, stearate, triolein, ethyl alcohol, glucose, glycerol, acetaldehyde, β-hydroxybutyrate, lactate, glycine, phenylalanine, histidine.

8. Dihydroxyacetone and phenylglyoxal are oxidised by very dilute hydrogen peroxide without peroxidase.

I wish to thank Sir F. G. Hopkins for his constant encouragement, and Prof. D. Keilin for his very kind interest in this work.

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III. THE CHEMISTRY OF OESTRIN.

V. THE MECHANISM OF THE CONVERSION OF TRIHYDROXYOESTRIN INTO KETOHYDROXYOESTRIN.

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In previous papers [Marrian 1930, 1, 2, 3] the isolation from the urine of pregnant women of an oestrus-producing crystalline substance characterised as a trihydroxy-compound of the formula $C_{18}H_{21}(OH)_3$ was described. Shortly before this Doisy *et al.* [1929], Butenandt [1929] and Dingemanse *et al.* [1930] had reported the isolation of an active crystalline substance from the same source, which from the melting-point and from the analytical data was clearly different from the compound referred to above. Butenandt [1930, 2] has satisfactorily shown that the second compound is a hydroxyketone of the formula $C_{18}H_{22}O_2$. Thayer, Veler and Doisy [1930] at about the same time suggested the formula $C_{18}H_{21}(OH)_2$ for this substance, but later [Thayer, Levin and Doisy, 1931, 2] accepted Butenandt's view that the substance was a hydroxyketone.

The suggestion is now made that, for the sake of convenience, the names *trihydroxyoestrin* and *ketohydroxyoestrin* should be provisionally adopted to designate these two active compounds. These names will therefore be used throughout this communication. The adoption of this system of nomenclature seems in many respects preferable to the use of the terms *theelol* and *theelin* that have been suggested by Doisy and his colleagues.

The suggestion was tentatively made [Marrian, 1930, 3] that ketohydroxyoestrin might be converted into trihydroxyoestrin by the uptake of one molecule of water at some stage during the process of purification of the crude urine extracts. It was however shown by Butenandt [1931, 1, 2] that both compounds existed together in urine and that ketohydroxyoestrin could be isolated from the mother-liquors of trihydroxyoestrin when the latter was prepared by the method described by one of the authors [Marrian, 1930, 3]. Doisy and Thayer [1931] have since also isolated trihydroxyoestrin as well as ketohydroxyoestrin from urine.

Butenandt [1931, 1, 2] has discovered the remarkable fact that, on sublimation in a high vacuum with potassium bisulphate, trihydroxyoestrin may be converted with loss of the elements of water into ketohydroxyoestrin identical with that isolated directly from urine extracts.

It was shown [Marrian, 1930, 2, 3] that one of the hydroxyl groups in trihydroxyoestrin was weakly acidic in character. Since the substance also gave colour reactions characteristic of phenols it was inferred that this acidic hydroxyl was phenolic. Similar views have since been expressed by Thayer, Levin and Doisy [1931, 1]. Ketohydroxyoestrin is also a weakly acidic substance, as Butenandt [1929] originally showed. Butenandt [1931, 1] suggested that the acidity was due to enolisation of the carbonyl group. Since the elimination of water from trihydroxyoestrin to yield ketohydroxyoestrin must involve the intermediate formation of such an enolic form, this suggestion appeared feasible. On the other hand, Thayer, Levin and Doisy [1931, 2] have suggested that the acidity is due to "acidic ionisation of the hydroxyl." Their evidence for this view is that ketohydroxyoestrin on methylation yields a monomethyl ether which is insoluble in alkali and which does not give a positive Millon reaction. They appear, however, to have overlooked the possibility that the group which can be methylated and the one responsible for the positive Millon reaction, might conceivably be the enolised carbonyl group postulated by Butenandt.

The work described in this paper was designed to settle definitely whether the acidity of ketohydroxyoestrin is due to the phenolic nature of the hydroxyl group, or whether it is due to enolisation of the carbonyl group. If the former view is correct, then in the conversion of trihydroxyoestrin to ketohydroxyoestrin, water must be eliminated from the two non-acidic hydroxyls. If the latter view is correct, then the phenolic hydroxyl in trihydroxyoestrin must be involved in the elimination of water to form dioxyoestrin. It is clearly not possible that the acidity of ketohydroxyoestrin is due to a phenolic hydroxyl and also to an enolised carbonyl group, since the monomethyl ether of ketohydroxyoestrin is insoluble in alkali.

The problem was easily and conclusively settled in the following manner. The monomethyl ether of trihydroxyoestrin was sublimed in a high vacuum with potassium bisulphate in the manner described by Butenandt for the conversion of trihydroxyoestrin into ketohydroxyoestrin. The product so obtained proved to be identical in melting-point, optical rotation, and crystalline form with the monomethyl ether of ketohydroxyoestrin. Clearly, therefore, water was eliminated from the two unmethylated alcoholic hydroxyl groups. As a further proof, it has been shown that ketohydroxyoestrin methyl ether and the dehydration product of trihydroxyoestrin methyl ether give identical oximes.

Experimental.

Methods.

Physiological tests of the potency of the compounds were carried out in the manner described in previous papers [Marrian, 1930, 1, 3].

Optical rotations were determined in a 0·5 dm. Fischer micro-tube, using the mercury-green line. Since the possible errors in such micro-determinations are considerable, the determinations have been done in duplicate where possible.

The melting-points recorded are uncorrected. In all cases they were carried out with short-stemmed Anschütz thermometers.

Micro-analyses were carried out by Dr A. Schoeller of Berlin. In all cases the substances were dried at 80° over phosphorus pentoxide in a high vacuum before combustion.

Preparation and purification of trihydroxyoestrin.

The trihydroxyoestrin used in this work was an accumulation from different batches worked up by different methods. The method of isolation described here is the one found by experience to yield the best results and incorporates several of the purification processes first described by Butenandt.

The urine was acidified with sulphuric acid and extracted four to six times with ether. The ethereal extract, after washing with water until free from acid, was evaporated and heated to 100° with 5 % aqueous potassium hydroxide for 2 hours. The alkaline mixture after saturation with carbon dioxide was thoroughly extracted with ether. The residue from the evaporation of this extract was boiled with dilute alcoholic hydrochloric acid [Butenandt, 1930, 1]. This acid mixture, after dilution with water, was extracted with ether, and the ethereal extract was washed with aqueous sodium carbonate solution several times. Phenolic substances were then removed from this ethereal extract by repeatedly shaking with aqueous potassium hydroxide. The aqueous alkaline solution was extracted with ether after saturation with carbon dioxide. It was found to be advantageous to extract this ethereal solution once more with alkali, since otherwise small quantities of non-phenolic unsaponifiable substances occasionally contaminated the final product. The final ethereal extract, after thorough washing with water, was evaporated to dryness and the residue was dissolved in 60 % aqueous ethyl alcohol. This solution was then extracted several times with benzene to remove the fraction containing the ketohydroxyoestrin. The benzene solution was washed with 50 % aqueous methyl alcohol, these washings being added to the main 60 % ethyl alcohol fraction [*cf.* Butenandt, 1931, 1]. The total aqueous alcoholic fraction after partial evaporation of the alcohol and dilution with water was extracted with ether, and the ethereal extract evaporated to dryness.

Crude trihydroxyoestrin was separated from this by dissolving in a small

volume of ethyl acetate and keeping at 0° for 24–48 hours. This crude material was cleaned by boiling in alcoholic solution with charcoal. The residue, after the evaporation of the alcoholic filtrate, was washed with a small volume of ice-cold ethyl acetate and dried. The product, when prepared in this way, had a melting-point usually between 276 and 280° and a physiological potency of 7–9 million mouse units per g.

The combined batches of crystalline material, after a second treatment with charcoal in alcoholic solution, were dissolved in *N* sodium hydroxide and filtered from small amounts of insoluble flocculent material. The alkaline filtrate was saturated with carbon dioxide and the precipitated trihydroxyoestrin was filtered off, washed and dried. The product was finally recrystallised from aqueous ethyl alcohol; M.P. 279·5–280·5° (slight decomp.).

4·930 mg. gave 13·550 mg. CO_2, 3·67 mg. H_2O,
C = 74·95 %, H = 8·27 %.
Calculated for $C_{18}H_{24}O_3$, C = 75·00 %, H = 8·33 %.

Optical rotation: $c = 2·58$ in pyridine, $\alpha = +0·554°$, $[\alpha]^{22}_{5461} = +42·9°$,
$c = 3·35$ in pyridine, $\alpha = +0·687°$, $[\alpha]^{23}_{5461} = +40·8°$,
$c = 0·432$ in alcohol, $\alpha = +0·159°$, $[\alpha]^{21}_{5461} = +78·3°$,
$c = 0·261$ in alcohol, $\alpha = +0·096°$, $[\alpha]^{22}_{5461} = +73·6°$.

Physiological potency: 7,630,000 m.u. per g.

Conversion of trihydroxyoestrin into ketohydroxyoestrin.

0·66 g. trihydroxyoestrin was mixed intimately with 16 g. of powdered fused potassium bisulphate and placed in a small hard glass retort of about 20 cc. capacity. The retort was heated in an air-bath for 5 hours at 110°, 3 hours at 140°, and finally 6 hours at 150°, at 0·02–0·05 mm. The contents of the whole retort were then washed out and suspended in a large volume of *N*/100 sodium hydroxide. The ketohydroxyoestrin was obtained from this mixture by repeated extraction with ether [*cf.* Veler, Thayer and Doisy, 1930]. The residue left on evaporation of the ethereal extract was washed twice with small volumes of ice-cold ether to remove traces of pigmented impurities, boiled in alcoholic solution with charcoal, and finally recrystallised from aqueous methyl alcohol. 0·22 g. of material melting with decomposition at 245–252° was obtained. The material was purified by sublimation for 20 hours at 150–160° and 0·01–0·02 mm. The final product melted at 242–246° with decomposition.

5·220 mg. gave 15·075 mg. CO_2, 3·880 mg. H_2O,
C = 78·75 %, H = 8·26 %.
Calculated for $C_{18}H_{22}O_2$, C = 80·00 %, H = 8·15 %.

Optical rotation: $c = 0·2675$ in ethyl alcohol, $\alpha = +0·201°$, $[\alpha]^{19}_{5461} = +188°$.

Physiological potency: 16,300,000 and 19,400,000 m.u. per g.

Preparation of trihydroxyoestrin methyl ether.

0·261 g. of trihydroxyoestrin was dissolved in 50 cc. $N/2$ sodium hydroxide and shaken for 2 hours with 2·5 cc. of dimethyl sulphate. The product was filtered off, suspended in 20 cc. $N/2$ sodium hydroxide, and warmed for 1 hour to dissolve all traces of unmethylated material. The insoluble residue was filtered off, washed thoroughly with water, and recrystallised once from aqueous ethyl alcohol and finally from absolute ethyl alcohol; M.P. 162·5–164°.

4·790 mg. gave 12·965 mg. CO_2, 3·77 mg. H_2O,
$C = 73·82\ \%$, $H = 8·74\ \%$.

3·080 mg. gave 2·355 mg. AgI (micro-Zeisel),
$(CH_3O-) = 10·09\ \%$.

Calculated for $C_{18}H_{23}O_2.OCH_3$, $C = 74·44\ \%$, $H = 8·86\%$, $(CH_3O-) = 10·26\ \%$.

Optical rotation: $c = 0·404$ in ethyl alcohol, $\alpha = +0·131°$, $[\alpha]^{19}_{5461} = +64·8°$,
$c = 0·298$ in ethyl alcohol, $\alpha = +0·098°$, $[\alpha]^{20}_{5461} = +65·8°$,
$c = 0·481$ in pyridine, $\alpha = +0·072°$, $[\alpha]^{20}_{5461} = +29·9°$.

Preparation of ketohydroxyoestrin methyl ether.

0·030 g. of ketohydroxyoestrin was dissolved with warming in 20 cc. $N/2$ sodium hydroxide and shaken for 2 hours with 0·5 cc. dimethyl sulphate. The product was filtered, and treated with a further quantity of $N/2$ alkali, as in the previous preparation. The washed residue, insoluble in alkali, was dried and recrystallised from absolute methyl alcohol after purification with charcoal in ethyl alcohol; M.P. 164–167°.

4·193 mg. gave 12·245 mg. CO_2, 3·200 mg. H_2O,
$C = 80·05\ \%$, $H = 8·48\ \%$.

3·066 mg. gave 2·650 mg. AgI (micro-Zeisel),
$(CH_3O-) = 11·40\ \%$.

Calculated for $C_{18}H_{21}O.OCH_3$, $C = 80·28\ \%$, $H = 8·45\ \%$, $(CH_3O-) = 10·91\%$.

Optical rotation: $c = 0·330$ in chloroform, $\alpha = +0·283°$, $[\alpha]^{21}_{5461} = +171°$,
$c = 0·440$ in chloroform, $\alpha = +0·377°$, $[\alpha]^{20}_{5461} = +171°$.

Dehydration of trihydroxyoestrin methyl ether.

0·104 g. trihydroxyoestrin methyl ether was intimately mixed with 2·5 g. of powdered fused potassium bisulphate and heated in a retort for 2 hours at 90° and 19 hours at 110–120° at 0·01 mm. The product which sublimed in the neck of the retort weighed 0·041 g. It was recrystallised twice from aqueous ethyl alcohol and finally from absolute methyl alcohol.

Optical rotation: $c = 0·552$ in chloroform, $\alpha = +0·446°$, $[\alpha]^{20}_{5461} = +171°$,
$c = 0·426$ in chloroform, $\alpha = +0·374°$, $[\alpha]^{20}_{5461} = +176°$.

It had M.P. 163–167°; mixed melting-point with ketohydroxyoestrin methyl ether, 162–168·5°. Crystals from absolute ethyl alcohol were similar in form to those of ketohydroxyoestrin methyl ether from the same solvent.

Preparation of the oxime of ketohydroxyoestrin methyl ether.

Ketohydroxyoestrin methyl ether was dissolved in ethyl alcohol and warmed for 2 hours with hydroxylamine hydrochloride and excess of sodium acetate. Excess of water was added and the precipitated oxime filtered off. The crude oxime was redissolved in alcohol and again precipitated with water; M.P. 182–187°.

A small amount of the trihydroxyoestrin methyl ether dehydration product was oximated in the same manner as described above; M.P. 183–187°; mixed melting-point with ketohydroxyoestrin methyl ether oxime, 180–184°.

SUMMARY.

1. Starting with trihydroxyoestrin ($C_{18}H_{24}O_3$; M.P. 279·5–280·5°; $[\alpha]_{5461}$ + 41·9° in pyridine, + 76° in ethyl alcohol; 7·6 million m.u. per g.) the following compounds have been prepared.

(*a*) *Ketohydroxyoestrin* ($C_{18}H_{22}O_2$; M.P. 242–246°; $[\alpha]_{5461}$ + 188° in ethyl alcohol; 17·8 million m.u. per g.) by distillation of trihydroxyoestrin with potassium bisulphate in a high vacuum.

(*b*) *Ketohydroxyoestrin monomethyl ether* (M.P. 164–167°; $[\alpha]_{5461}$ + 171° in chloroform) by treatment of ketohydroxyoestrin with dimethyl sulphate in alkaline solution.

(*c*) *Trihydroxyoestrin monomethyl ether* (M.P. 162·5–164°; $[\alpha]_{5461}$ + 29·9° in pyridine, + 65·3° in ethyl alcohol) by treatment of trihydroxyoestrin with dimethyl sulphate in alkaline solution.

(*d*) *Dehydration product of trihydroxyoestrin methyl ether* (M.P. 163–167°; $[\alpha]_{5461}$ + 173° in chloroform) by distillation of trihydroxyoestrin methyl ether with potassium bisulphate in a high vacuum.

(*e*) *Oxime of ketohydroxyoestrin methyl ether* (M.P. 182–187°).

(*f*) *Oxime of dehydration product of trihydroxyoestrin methyl ether* (M.P. 183–187°).

2. Compounds (*b*) and (*d*) had similar crystalline forms, the same melting-point, a mixed melting-point showing no depression and the same optical rotation. They are therefore identical.

Compounds (*e*) and (*f*) had the same melting-point and gave a mixed melting-point showing practically no depression. They are, therefore, identical.

3. These data are considered to provide conclusive evidence that in the conversion of trihydroxyoestrin to ketohydroxyoestrin water is eliminated between the two non-acidic hydroxyls, and that the acidity of the latter compound is due to a phenolic hydroxyl and not to enolisation of the carbonyl group.

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Note added February 1st, 1932. Since this work was completed, a short paper by Butenandt (*Z. angew. Chem.* **44**, 905) has appeared, in which he shows that the acidity of ketohydroxyoestrin is due to the hydroxyl group, since its esters are insoluble in alkali while derivatives involving the carbonyl group retain their solubility in alkali.

IV. THE SPERMICIDAL ACTIVITY OF QUINONES AND QUINOLS.

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Introduction.

In the course of his investigation [Baker, 1930, 1931] into the spermicidal effect of various substances Dr J. R. Baker invited me to collaborate with him in the examination of the action of pure chemical compounds on guinea-pig spermatozoa. By mutual agreement, many results of the biological tests of this joint work have been published elsewhere (Baker, 1932; in the press), but are unaccompanied by any comment on their significance from the chemical standpoint, although Dr Baker mentioned there that an article by me on this question was in preparation.

It is one purpose of this preliminary communication to mention the results of some further tests which Dr Baker has made and kindly permits me to quote. Its chief intention, however, is to discuss briefly possible modes of action of quinones and related compounds on the components of animal or vegetable tissues. An inspection of the relevant literature reveals that in the past only one of the many possibilities has been entertained—namely, the interaction of quinones with the primary amino-groups of proteins or their decomposition products, either as such, or as constituents of bacteria. Admittedly I have indulged in considerable speculation, but this is unavoidable in attempting to outline the scope of the problem in a preliminary communication. My thanks are due to Dr Baker for allowing me to include in this discussion results which he obtained before I joined him.

I take this opportunity to state that my main interest in this subject is not concerned with the contraceptive possibilities of the results obtained. It appears to me, however, that spermatozoa, by reason of their motility and of the spatial arrangement of their chemical components, are excellent material for the study of the effect and mode of action of chemical substances on the vitality of cells.

Technique employed.

The details of the technique have been amply elaborated by Baker in previous publications. The term "killing concentration" used in them and in this paper is the least concentration (weight/volume) in the series 1 : 50,

1 : 100, 1 : 200, 1 : 400, 1 : 800, *etc.*, which suffices to immobilise in 30 minutes in four consecutive experiments all guinea-pig sperms suspended in a glucose-saline fluid buffered at p_H 8·1 at 37°.

Results of Biological Tests.

The results to be described deal with two main groups of compounds—namely, quinol derivatives and phenolic aldehydes—but others have been included on account of their chemical relationship to these groups. The figures in brackets indicate the killing concentrations, and a list is appended showing the compounds mentioned in order of their lethality.

Quinol derivatives.

In studying the toxicity of chemical substances to cells, it is natural to turn attention in the first place to the phenols on account of their germicidal properties. In the case of spermatozoa, phenol (1 : 200) is relatively inactive. From the few experiments performed, it would appear that the introduction of carbon side-chains into the molecule increases the lethality, since a mixture of cresols (tricresol) (1 : 400) is more active than phenol, but is surpassed by thymol (1 : 3200). In this respect there is a parallel with the increase in germicidal values of the homologous phenols as compared with that of the parent substance [Morgan and Cooper, 1912; Tilley and Schaffer, 1926, 1927].

The simple dihydroxybenzenes show striking variations depending on the orientation of the hydroxyl groups. Quinol (1 : 6400) is much more active than catechol (1 : 200), which in turn is more effective than resorcinol (1 : 100). It should be noted that hexylresorcinol (1 : 6400) shows enhanced efficiency as compared with resorcinol, presumably for the same reason that it is more germicidal [Johnson and Lane, 1921; Leonard, 1924, 1925; Elliot and Barbour, 1925]. The monomethyl ethers of dihydroxyphenols—quinol monomethyl ether (1 : 100) and guaiacol—are no more reactive than phenol, which suggested that a polyhydroxylic compound might have some specific action.

Attention was then directed to the isomeric trihydroxybenzenes. Phloroglucinol and pyrogallol (1 : 50) are practically inactive, whereas hydroxyquinol (1 : 6400) is rather more efficient than quinol. This superiority of quinol and hydroxyquinol at once focused interest on the group of *p*-dihydroxybenzenes, and noteworthy results were obtained—methoxyquinol (1 : 25,600), methylquinol (1 : 25,600), butylquinol (1 : 12,800). 2 : 6-Dimethoxyquinol, however, shows a decreased activity. This observation, coupled with the fact that the tests are carried out in an aerated solution at about p_H 8 in which oxidation of quinols might be expected to occur [*cf.* La Mer and Rideal, 1924; Euler and Brunius, 1928], led to the investigation of the *p*-quinones. These were all more active than the corresponding quinols, and out of nine tested, six—toluquinone, butylquinone, methoxyquinone,

ethylquinone, benzoquinone, *p*-xyloquinone (all 1 : 51,200)—were more spermicidal than mercuric chloride, hitherto the most active agent known.

The disubstituted quinones, 2-methoxy-5-methylquinone (methoxytoluquinone) (1 : 6400) and thymoquinone (1 : 12,800), were less lethal than those just mentioned, as also was duroquinone (no effect at 1 : 25,600), in which all four nuclear hydrogens are replaced by methyl groups. Unfortunately, the sparing solubility of duroquinone in water prevented its being tested at higher concentrations.

A few quinonoid dyestuffs—malachite green, crystal violet, pyronine G—and also nitrobenzene and dinitrobenzene were examined, but of these only malachite green (1 : 12,800) proved relatively efficient.

In view of the facile oxidation of *p*-phenylenediamine to benzoquinone which can be effected in the chemical laboratory, tests were carried out with this diamine (slight activity at 1 : 100) and with the *ortho*- (1 : 50) and *meta*- (fails at 1 : 50) isomerides. It must be concluded that no such oxidation occurs under the conditions of the biological test, and that whatever may be the explanation for the high spermicidal activity of the quinones and quinols it does not hold for the diaminobenzenes.

Table I. *Killing concentrations.*

Substances are arranged in order of activity, except where they are bracketed together. In the section "not fully tested," 3 indicates full activity of sperms, 0 indicates all immobile. Intermediate activity is graded 1, 2, *etc.*

1 : 51,200
- Toluquinone
- Butylquinone
- Methoxyquinone, Ethylquinone
- Benzoquinone
- *p*-Xyloquinone

1 : 25,600
- Mercuric chloride
- Methoxyquinol
- Formaldehyde, Paraformaldehyde
- Methylquinol

1 : 12,800
- Butylquinol
- Malachite green
- Thymoquinone

1 : 6,400
- 2-Methoxy-5-methylquinone, Hexylresorcinol
- 2 : 6-Dimethoxyquinol
- Hydroxyquinol
- 2-Hydroxy-3-ethoxybenzaldehyde (ethoxysalicylaldehyde), 2-Hydroxy-3-methoxybenzaldehyde (*o*-vanillin)
- Quinol

1 : 3,200
- Salicylaldehyde
- Crystal violet
- Thymol

1 : 400
- Tricresol

1 : 200
- Catechol
- Phenol

1 : 100
- Quinol methyl ether, Acetaldehyde
- Chloral hydrate, Resorcinol

1 : 50
- Pyrogallol
- *o*-Phenylenediamine

Fail to kill at 1 : 50
- *m*-Phenylenediamine

Not fully tested.
- Duroquinone. No effect at 1 : 25,600
- Nitrobenzene, Dinitrobenzene, *p*-Nitrosodimethylaniline, Pyronine G — No effect at 1 : 6400
- Guaiacol 3 at 1 : 1600
- *iso*Vanillin 3 at 1 : 1600
- *p*-Dimethylaminobenzaldehyde 3 at 1 : 1600
- *p*-Hydroxybenzaldehyde 2+ at 1 : 1600
- *p*-Phenylenediamine 1, 2, 2, at 1 : 100
- Phloroglucinol 1, 1, 1, at 1 : 400
- Not sufficiently soluble to try at 1 : 200
- Phloroglucinaldehyde 0, 0, 1 at 1 : 1600
- Vanillin 0 at 1 : 400, 3 at 1 : 1600

Phenolic aldehydes.

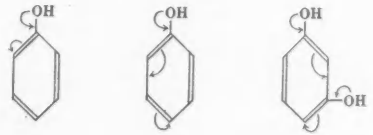
The high toxicity of formaldehyde (1 : 25,600) is striking when compared with that of acetaldehyde and chloral (both 1 : 100), and is doubtless analogous to its vigorous bactericidal action.

The aromatic aldehydes also exhibit marked divergences in activity. Salicylaldehyde, methoxysalicylaldehyde (*ortho*vanillin), and ethoxysalicylaldehyde (all 1 : 6400) are about sixteen times as effective as vanillin, *iso*-vanillin, *p*-hydroxybenzaldehyde, and *p*-dimethylaminobenzaldehyde, all of which fail to kill at 1 : 1600, and are probably lethal at 1 : 400. It is interesting to note that the *o*-phenolic aldehyde phloroglucinaldehyde is intermediate in character, being somewhat more active than the last mentioned group, though inferior to the substituted salicylaldehydes.

Discussion.

In the action of chemical substances on living cells it is evident that many factors may play a simultaneous part in producing the final result. For example, the effects due to alterations in surface tension, permeability of tissues, adsorbability and other similar physical causes may entirely outweigh those due to specific chemical groups. It would therefore be dangerous to contrast the behaviour of standard material under conditions which were changed only by the addition of different chemical substances in varying proportions, unless a close relationship existed not only between the radicals present, but also in the molecular size of each compound. The members of the group of quinones and quinols described above are probably free from any significant variations in these respects, and consequently a chemical explanation may be sought of their vigorous spermicidal properties as compared, for example, with those of the phenols and resorcinols. In order to do this, it will be necessary to consider briefly the modern conceptions of reactivity of such compounds.

In the terms of the electronic theory of the mechanism of reactions [Robinson, 1925; Rây and Robinson, 1925; Allan, Oxford, Robinson and Smith, 1926; Lapworth and Robinson, 1928], the reactivity of phenol is expressed by either of the formulae I or II. In these a definite polarisation of the molecule



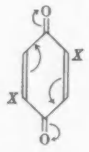
(I) (II) (III)

has occurred owing to the tendency of the oxygen of the hydroxyl group to increase its covalency with the neighbouring carbon atom. This effect is carried through the conjugated system of the benzene ring, and results in the

production at the *o*- and *p*-positions of an excess of electrons as compared with the non-polarised state. The carbon atoms at these positions are termed anionoid centres, and are attacked by kationoid, or electron-seeking, reagents.

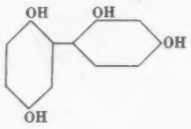
The case of resorcinol is merely an elaboration of that of phenol. The individual effects of the two hydroxyls are combined (III), and the intense sensitivity of resorcinol to kationoid reagents is well known.

As regards quinones, however, the situation is reversed. On the same theory the properties associated with doubly-linked oxygen are explained by its tendency to decrease its covalency by attracting electrons away from the adjacent carbon atom. The oxygen atoms of a quinone thus become anionoid centres, and the electron drift is carried on through the two conjugated systems of which benzoquinone, for example, is composed (IV and V). The carbon atoms *X* and *Y* in the β-positions to the carbonyl groups are thus the kationoid centres, and will be attacked by anionoid, or electron-donating, reagents.



(IV)

(V)



(VI)

The following, among many other possible illustrations, will serve to indicate the great differences between the two types of reactivity. Phenol is very readily substituted by bromine and by dilute nitric acid: benzoquinone takes up bromine additively [Sarauw, 1881; Nef, 1890] and is not attacked by cold, fuming nitric acid [Liebermann, 1877; Nietzki, 1882]. On the other hand, benzoquinone and resorcinol readily react when heated together [Pummerer and Huppmann, 1927], the anionoid centre of resorcinol combining with the kationoid centre of the quinone to yield tetrahydroxydiphenyl (VI). No such reaction takes place between phenol and resorcinol.

In virtue of this property of reacting with electron-donating reagents, the quinones combine chemically with many different types of compounds, the union in many cases being extremely facile [see Houben, 1923]. It is of interest to discuss here only their behaviour with substances or groups which may reasonably be expected to occur in living tissues. It should be noted, however, that the omission of a particular group, or type, does not exclude the possibility that it may, as a component of the spermatozoon, undergo reaction with a quinone.

Quinol derivatives.

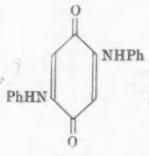
The reactivity of quinols as a class is phenolic in character. Nevertheless, it will be observed that the quinols are, in general, only slightly less active than the corresponding quinones. It seems probable, therefore, that they exercise their spermicidal properties not by precipitation of cell colloids as do

phenols and allied germicides, but by either (i) undergoing oxidation to the quinones by oxygen in the alkaline fluid (*cf.* Harger [1922, 1924] on the action of primary and secondary aliphatic amines on quinol), or (ii) by interfering with the oxidising systems of the cell.

Reactions of p*-quinones.*

Primary amines. Primary, secondary and tertiary aromatic amines form highly coloured additive complexes with quinones [Jackson and Clarke, 1905; Fecht, 1908; Schlenk and Knorr, 1909; Siegmund, 1910; Brass, 1913; Suchanek, 1914; Kremann *et al.*, 1922; Hixon, 1923]. When those derived from primary amines are oxidised by aerial oxygen, by a molecule of a quinone or by other means, they are easily converted into nuclear condensation products, of which dianilinoquinone (VII) is typical. In the interaction of quinones with aliphatic primary amines, however, the first isolable product is the corresponding alkylaminoquinone [Fichter and Willmann, 1904; Fichter, 1908; Zincke, 1881].

In extending these foregoing results to animal tissues, Fischer and Schräder [1910] prepared the substance (VIII) from benzoquinone and glycine ester, and observed that toluquinone formed a similar compound. Hilpert [1925] selected glycine anilide to represent a peptide, and found that one molecule only entered the nucleus of benzoquinone (IX), toluquinone and α-naphthoquinone respectively.



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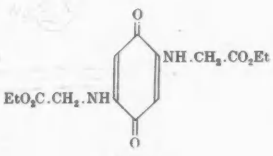

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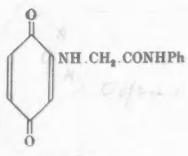


(IX)

(X)

No definite compounds have been isolated as the result of the action of quinones on proteins or their decomposition products, apart from amino-acids. Several investigators, however, have been attracted by the similarity between the red colours of the anilinoquinones and related compounds and those produced when quinones react with proteins, peptones or amino-acids, and have associated the production of colour with the formation of substituted aminoquinones from the primary amino-groups of these substances [Raciborski, 1906; Scharvin, 1913; Suida, 1913; Cooper, 1913; Morgan and Cooper, 1921]. The high bactericidal value of the quinones has been studied at some length on the basis of this type of action by the germicide on the primary amino-groups not only of the complex proteins, but also of simple water-soluble cell constituents [Morgan and Cooper, 1924; Hilpert, 1925; Cooper and Nicholas, 1927; Cooper and Haines, 1928, 1929].

Cooper [1913] and Morgan and Cooper [1921] have suggested that compounds analogous to quinonedianil (X) may result from interaction of primary amino

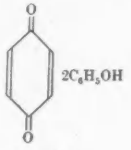
groups of cell constituents and quinones. This view is largely based on the fact that gelatin is not attacked by an aqueous solution of quinone dioxime, whereas after treatment with quinone it may be isolated in a coloured state having altered physical properties. The evidence for this type of reaction seems somewhat inadequate. A study of the literature shows that in general nuclear condensation is preferred to "anil" formation, and that quinone dioxime does not retain the high nuclear reactivity of the parent substance. This may be deduced on theoretical grounds from electronic considerations, and it is clearly unsatisfactory to compare the reactivity of the two substances.

As mentioned above, the possibility that groups, other than primary amino-groups, of cell constituents may enter into combination with quinones does not appear to have been entertained. It is noteworthy, however, that Cooper and Haines [1928] observed in a quantitative experiment that the amount of benzoquinone taken up by gelatin is considerably in excess of that required by the primary amino-groups. Since the nitrogen of peptide groups does not react with quinones, the additional amount of benzoquinone is regarded by them as being adsorbed and not chemically combined, but it seems not improbable that entry into chemical union may account for some at least of the "adsorbed" quinone in their experiment. Further, Suida [1913] found that 2 : 5- and 2 : 6-xyloquinone and thymoquinone do not dye wool, whereas benzoquinone, toluquinone and others do so, and concluded that these dialkylquinones lacked the power of forming aminoquinonoid derivatives. Reference to Table I shows that two of these are excellent spermicides and may therefore have the power of entering into other types of compounds than those which benzoquinone forms with wool. These facts suggest that reactions such as are described below may play as important a part in the action of quinones on cell substances as do those in which primary amino-groups participate.

Secondary amines. These are known to occur in animal tissues, and there is ample evidence that they react chemically with quinones to produce substituted aminoquinones, analogous in constitution to tetramethyldiaminobenzoquinone [Mylius, 1885; Harger, 1922]. In the case of reduced heterocyclic compounds containing imino-groups, Schmidt and Sigwart [1913] have recorded the production of aminoquinones and the examples quoted, *e.g.* piperidine, have analogies with the constituents of animal material. These authors and Möhlau and Redlich [1911] observed that pyrrole and α-methylindole readily react with quinone, whereas carbazole and tetrahydrocarbazole do not; the suggestion made by Möhlau and Redlich that carbon-to-carbon condensation has occurred here would seem to be correct and probably limits the possibility of this type of combination to pyrroles having a free α- or β-position, unless it can be shown that indoles occur naturally in which the β-position is unsubstituted.

Phenoquinones and related substances. The additive complexes which quinones form with aromatic primary, secondary and tertiary amines have already been mentioned, but it is very doubtful if this type of combination would play

a part in the action of quinones on spermatozoa. Additive complexes, however, are formed with great ease also when quinones react with phenols, two hydroxyl groups, in general, uniting with one molecule of the quinone. Very many cases of such phenoquinones (XI) are known [Beilstein, 1925], quinhydrone (XII) being a special example, and the occurrence of phenolic groups in living matter has long been established. Quinhydrone is stable up to p_H 8 [La Mer and Parsons, 1923], and complexes of this type would thus appear to be possible within the p_H limits of the living cell and the glucose-saline fluid.



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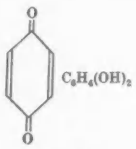
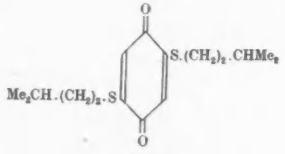


(XII)

The conversion in the cell of phenoquinones into diphenylquinones is possible, but improbable, since in the laboratory it involves the use of aluminium chloride as a condensing reagent [Pummerer and Prell, 1922]. Resorcyl groups, being highly efficient as anionoid reagents (see above), might conceivably react in this way, but their presence in animal, as opposed to vegetable, organisms has not been demonstrated.

Sulphydryl compounds. Thiophenols and mercaptans unite readily with benzoquinone and toluquinone, forming thiophenoquinones—additive complexes similar to the phenoquinones. When these are oxidised or undergo autoxidation they readily yield substituted thiolquinones, of which di-*iso*amylthiolquinone (XIII) is typical [Tarbouriech, 1901; Posner, 1904; Posner and Lipski, 1904].



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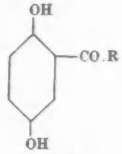

(XIV)

The importance of the part played by sulphydryl groups in living processes is fully recognised, and it is probable that the thioliminazoles would react with quinones in a similar manner from their analogy with the thiophenols.

Aldehydes. When aqueous solutions of *p*-benzoquinone and acetaldehyde, *iso*valeraldehyde or benzaldehyde are exposed to light, a dihydroxyphenylketone (XIV) is produced [Klinger and Standke, 1891; Klinger and Kolvenbach, 1898]. With these results in view, it is noteworthy that the presence of aldehydic groups has been proved or suspected in the intermediates of animal

processes and in enzymes, *e.g.* erepsin [Josephson and Euler, 1927; Waldschmidt-Leitz and Rauchalles, 1928].

Alcohols. A few examples are known in which quinone reacts with alcohols to form ethers of 2 : 5-dihydroxyquinones [Möhlau and Klopfer, 1899; Knoevenagel and Bückel, 1901]. The reaction, however, is in general not facile and requires a condensing agent.

Oxidation-reduction. The ease with which quinones can enter into oxidation-reduction systems is fully established [Oppenheimer, 1926]. Wieland and Bertho [1928], Neuberg and Simon [1926] and Lüers and Mengele [1926] found that quinones can act as hydrogen acceptors in acetic or alcoholic fermentations, and Dixon [1926] observed that quinone played a similar part in the xanthine oxidase system. By *in vitro* experiments Wieland and Bergel [1924], Bach [1914] and Traube [1911] showed that *p*-benzoquinone transformed α-amino-acids to aldehydes, and Ciamician and Silber [1901] used it to oxidise alcohols to aldehydes, polyhydric alcohols to aldoses (ketoses), and glucose to glucosone.

The effect of quinones on sulphydryl-disulphide systems does not appear to have been investigated, although it might be anticipated from a comparison of oxidation-reduction potentials [Michaelis, 1929] that they would maintain the systems in an oxidised state. Posner and Lipski [1904] have observed that xyloquinone, in which the nuclear activity is somewhat decreased by the substituents (see later), oxidises thiophenol to diphenyldisulphide.

It is well recognised that quinol is dehydrogenated by enzymes, as for example by muscular tissue [Wieland and Lawson, 1931; see also Wieland and Sutter, 1928; Battelli and Stern, 1912], and it has in addition remarkable antioxidant properties [Moureu and Dufraisse, 1922, 1, 2; 1928].

Enough has been indicated above to show that the high spermicidal values of *p*-quinones and quinols can receive abundant explanation, whether their action is regarded as chemical union with cell constituents, or as interference with vital enzymic processes, and it is hoped that further investigations will allow definite interpretations to be placed on their modes of action.

ortho*Quinones and* ortho*quinols.*

It may be asked why catechol is so much less active than quinol since much of what has been said undoubtedly would apply, and in many cases does apply, to it and to *o*-benzoquinone. The explanation of this unexpected variation is probably to be found in the greater reactivity of *o*-benzoquinone and catechol as compared with the *p*-isomerides. In the alkaline media in which the test is carried out, *o*-benzoquinone would be extremely rapidly polymerised to complex inactive substances on account of its instability [Willstätter and Pfannenstiel, 1904], whereas *p*-benzoquinone is comparatively much less labile [Meunier and Queroix, 1925].

Phenolic aldehydes.

It seems reasonable to assume that the phenolic aldehydes which have been tested form a group in which the physical effect of each member on the cell should not differ very greatly. Here again as with the quinones and related compounds, it is possible to search for a chemical explanation of activity.

The moderately high lethality of salicylaldehyde and its vicinal methoxy- and ethoxy-derivatives suggested a hypothesis which related the action of these aldehydes with that of the quinones. Dakin [1909] showed that the sodium salts of *o*- and *p*-hydroxyaldehydes of the benzene series are readily and often almost quantitatively oxidised to the corresponding *o*- and *p*-dihydroxybenzenes by hydrogen peroxide. The same author [1922] has drawn attention to the similarity between hydrogen peroxide oxidations *in vitro* and many oxidations *in vivo*, and it seemed possible that the *o*-hydroxyaldehydes were in themselves little reactive, and that the spermatozoa by this oxidation brought about their own extermination. This hypothesis became untenable, however, when it was found that vanillin is about sixteen times less active than *o*-vanillin. From the experimental results it seems possible to correlate spermicidal activity with the adjacent orientation of the hydroxyl and aldehydic groups, but further discussion is reserved for a future publication.

COMPARISON OF THE SPERMICIDAL AND GERMICIDAL POWERS OF *p*-QUINONES AND THE CORRELATION OF SPERMICIDAL AND CHEMICAL ACTIVITY.

In conformity with previous work, the killing concentrations quoted in this paper are calculated on equal, and not equimolecular, weights of chemical substances. This is unfortunate from the point of view of an absolute experimental comparison of the spermicidal powers of different members of the quinone, and quinol, groups. From a consideration of molecular weights, however, and from the protocols of experiments, it is possible to arrive at the following series of *p*-quinones in order of spermicidal activity:

Toluquinone $\doteqdot$ butylquinone $>$ methoxyquinone $\doteqdot$ ethylquinone $>$ *p*-benzoquinone $\doteqdot$ *p*-xyloquinone $>$ thymoquinone $>$ 2-methoxy-5-methylquinone.

The experimental accuracy of the technique employed does not allow a definite decision to be reached between toluquinone and butylquinone. As an illustration of their superiority over *p*-benzoquinone, however, it may be mentioned that toluquinone is very nearly twice as spermicidal as benzoquinone. Similarly it is not possible to make a definite decision between methoxyquinone and ethylquinone, nor between benzoquinone and *p*-xyloquinone. The first six members of the series lie comparatively close together, and are separated by an interval from thymoquinone, which in turn is followed at some distance by methoxytoluquinone.

It is evident that monoalkylquinones are more spermicidal than benzoquinone, and that disubstituted quinones are less spermicidal. It should be

noted that the substituents have been selected so that they are themselves unlikely to exert any influence through chemical reaction with the cells. The case of methoxyquinone is a special one, and will be discussed below.

These results are strikingly different from those of Cooper and his collaborators on the germicidal powers of quinones; they find, in general, that bactericidal activity is diminished on ascending the homologous series.

Erdtman [1932][1] has recently conducted a systematic investigation into the chemical reactivity of a number of *p*-benzoquinones. As a test of their reactivity to anionoid reagents, he has examined under standard conditions the ease with which they are converted into triacetoxy-derivatives by acetic anhydride and sulphuric acid [Thiele, 1898], and has also studied the readiness with which they yield the corresponding quinols with sulphur dioxide. He finds that the following series holds,

methoxyquinone > toluquinone > *p*-benzoquinone > *p*-xyloquinone > *m*-xyloquinone > 2 : 6-dimethoxyquinone > 2 : 5-dimethoxyquinone,

and considers that the superiority of the monosubstituted quinones over benzoquinone is due to the neutralisation by the substituent of the electronic attraction of the carbonyl group, whereas in the disubstituted quinones the electronic effects of both carbonyl groups are decreased. In both cases the magnitude of the neutralisation is dependent on the strength of electronic repulsion of the substituent.

The striking similarity between the spermicidal and chemical activities of the *p*-quinones lends great support to the views which have been expressed above as to the reasons for their lethality. The only anomalous result is that of methoxyquinone, which is the most reactive chemically, but is only slightly greater than benzoquinone in spermicidal effect. The explanation is simple; so reactive is this quinone chemically that during the course of the biological test it undergoes polymerisation to a considerable extent at the alkaline p_H of the glucose-saline buffer solution.

Again taking molecular weights into account, the group of quinols may be arranged in order of their lethality to spermatozoa—methoxyquinol > methylquinol ≒ butylquinol > 2 : 6-dimethoxyquinol > hydroxyquinol > quinol. The similarity of the first part of the series to the corresponding portion of the quinone series is striking, and suggests that the quinols may exert their spermicidal action in the form of quinones by undergoing gradual oxidation in the alkaline fluid. Hydroxyquinol, which should in theory resemble methoxyquinol, probably takes a low place on account of its instability in alkaline solution, and, as compared with the position of benzoquinone in the quinone series, quinol is less active than might be expected. This result might conceivably receive an explanation in the rates of oxidation of the quinols, and the question is being investigated.

[1] I wish to express to Dr H. Erdtman my indebtedness to him not only for allowing me to quote these results, but also for giving me the opportunity on various occasions of discussing with him questions relating to the chemical reactivity of quinones.

PREPARATION OF CHEMICAL SUBSTANCES.

I am greatly indebted to Dr H. Erdtman, Dr Wilson Baker, Dr S. Sugasawa and Mr T. H. Mead for gifts of some of the compounds which have been tested. The following compounds have not previously been described.

2 : 5-*Dihydroxyphenyl propyl ketone.* Powdered aluminium chloride (30 g.) was added gradually to an ice-cold solution of quinol dimethyl ether (30 g.) and *n*-butyryl chloride (23 g.) in carbon disulphide (30 cc.). After 12 hours, ice was added, and the mixture was steam-distilled to remove solvent and traces of quinol dimethyl ether. The product of the reaction, 2 : 5-dimethoxyphenyl propyl ketone, was not readily volatile in steam, and was extracted from the residue with ether, dried, and obtained as a brown oil by evaporation of the solvent. Without further purification, this ketone (30 g.) was demethylated by being heated under reflux with a boiling mixture of concentrated hydrobromic acid (100 cc.) and glacial acetic acid (100 cc.) for 18 hours. The liquid was poured into water, and the product extracted with ether, freed from acids by being shaken with aqueous potassium bicarbonate solution, and obtained in the crystalline state when the solvent was evaporated. 2 : 5-Dihydroxyphenyl propyl ketone was purified by distillation, B.P. 193°/16 mm., followed by crystallisation from carbon tetrachloride. It formed yellow prismatic tablets, M.P. 87–89°, which changed to a powdery state shortly before melting, and dissolved readily in benzene and chloroform. (Found: C, 66·5; H, 6·6. $C_{10}H_{12}O_3$ requires C, 66·7; H, 6·7 %.) The preparation of this substance by heating together quinol, *n*-butyryl chloride and zinc chloride is less satisfactory. The method described has been used with equal success for the preparation of 2 : 5-dihydroxyacetophenone, but this was accompanied by a small amount of a deep-red by-product, which, on treatment with sodium acetate and hydroxide, exhibited the colour changes associated with an anthocyanidin.

2 : 5-*Dihydroxybutylbenzene* (butylquinol). The ketone described above (10 g.) was reduced according to Clemmensen's method with amalgamated zinc and equal volumes of concentrated hydrochloric acid and water. After 10 hours, the hot solution was filtered, cooled, and extracted with ether. The extract, when washed, dried and distilled, yielded a brown oil which was fractionated at reduced pressure. Butylquinol passed over at 180–185°/20 mm., and crystallised from carbon tetrachloride in small, colourless needles, M.P. 89°. (Found: C, 73·2; H, 8·6. $C_{10}H_{14}O_2$ requires C, 72·3; H, 8·4 %.)

Butyl-1 : 4-*quinone.* Butylquinol was dissolved in hot water, and the solution was quickly cooled so that no separation of solid occurred. A large excess of aqueous ferric chloride solution was added, and butylquinone separated immediately as a yellow oil which rapidly crystallised. It was collected, dried in a vacuum desiccator, and purified by sublimation at 100°, since it was readily soluble in all the usual organic solvents including light petroleum. It formed orange-yellow needles, M.P. 32°, which were very rapidly attacked by 2*N* sodium hydroxide. (Found: C, 72·9; H, 7·3. $C_{10}H_{12}O_2$ requires C, 73·1;

H, 7·3 %.) On being kept, the quinone was transformed into a dark-coloured oil in the course of 24 hours; from this, some unchanged quinone could be extracted by means of light petroleum.

Summary.

(1) *p*-Quinones and quinols are highly toxic to guinea-pig spermatozoa.

(2) Possible reasons for their great toxicity, as compared with that of monohydroxyphenols and resorcinol derivatives, are discussed on the basis of chemical interaction of the spermicide with cell constituents, and an explanation is suggested for the relative lack of toxicity of *o*-benzoquinone and catechol.

(3) A series of *p*-quinones arranged in order of spermicidal efficiency resembles very closely a series of the same, or similar, quinones placed in order of chemical activity.

(4) Monoalkyl-*p*-quinones are more spermicidal than *p*-benzoquinone, which is more toxic than dialkyl-*p*-quinones.

(5) Aromatic *o*-hydroxyaldehydes are much more spermicidal than *m*- or *p*-hydroxyaldehydes.

My thanks are due to Dr J. R. Baker for his invitation to collaborate with him in this work, and for carrying out the numerous biological tests.

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V. THE SWELLING OF SINGLE COLLAGEN FIBRE-BUNDLES.

By ROBERT HENRY MARRIOTT.

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(*Received December 9th, 1931.*)

Kaye and Jordan-Lloyd [1924] in an investigation of the swelling of fibrous tissue showed that single teased-out collagen fibres altered in length and breadth when treated in solutions of acids and alkalis. In that work single fibre-bundles were obtained and the length and breadth were determined under the microscope by means of an eyepiece micrometer. The fibre-bundles were then treated with various reagents and the fibre-bundle re-measured. This method of observation is, however, extremely tedious and it is complicated by the great difficulty of measuring the breadth of the fibre-bundle, since this can vary to a very marked degree along the length of the bundle. This trouble becomes almost insurmountable when the fibre-bundles are swollen in acid solution, since there is a large amount of distortion and any attempt to express the results numerically leads to large errors.

It was also shown that the swelling of hides and skins in the various liquors was dependent on the degree to which the hide fibres had been freed from each other. Thus the increase in volume of hide powder was of the same order as that of gelatin, while in the cases of pieces of hide and skin the percentage increase, although running parallel with that of hide powder, was very much smaller. The obvious difference between hide, as such, and hide powder is that in the former the individual fibres are not free to contract along their length nor are they free to increase in width since they are more or less closely interwoven. Thus the results obtained with loose fibre-bundles are not truly comparable with the effect produced on pieces of skin.

It was therefore decided to endeavour to follow more accurately the effect of various solutions on single collagen fibre-bundles which were lightly held by their ends so that the effects observed would be more comparable with those taking place in the fibres when hides and skins are treated with different reagents. The first experiments were directed towards following the swelling of the fibres in acid liquors of known p_H value.

Experimental.

In the preliminary experiments, single bundles of purified collagen (acetone-dried), obtained from ox hide, were teased out and fastened at the ends to a glass slide by means of paraffin wax. The fibre-bundle was then soaked in distilled water, which was afterwards replaced by $N/100$ hydrochloric acid. The fibre swelled in the acid but it was not distorted, neither was there any marked indication of the constricting elements of reticular tissue which encircle the fibres and which are generally to be observed when free single fibre-bundles are swollen in dilute hydrochloric acid. In a further experiment, an attempt was made to cement the fibre-bundle to two fine glass rods, one of which was fixed and the other suspended from a balance so that an increased load could be applied to the fibre-bundle. It was found that when there was no load on the fibre-bundle the characteristic contraction in length and increase in width which occurs when collagen fibres are treated with $N/100$ hydrochloric acid could be observed, and the constricting elements were visible. When a load was applied, the fibre increased in length and decreased in thickness, the constricting elements becoming less and less apparent. Loads of 7 g. generally brought about the total suppression of the constricting effect and loads of 7 to 10 g. generally caused the fibre-bundles to break. The breaking was similar to that of an ordinary worsted yarn, the individual fibres sliding past each other, the two broken ends being frayed. The width of the different fibre-bundles used in these experiments varied between about 0·10 and 0·30 mm.

Owing to the difficulty of cementing the fibres with paraffin wax, all the later experiments were carried out by holding the fibre-bundles by means of two gold pen nibs. One of the nibs was fixed to a glass slide by means of plasticine and the other was allowed to hang freely, thus putting a constant load on the fibre. The glass slide could then be placed in a rectangular trough containing the experimental liquor and the fibre-bundle viewed through a microscope.

The fibre-bundle was treated with distilled water and its length measured by means of an eyepiece micrometer. This length was divided by five and the width of the fibre-bundle was determined at each fifth of its length. The average width was then calculated and from these two values the approximate volume of the fibre-bundle was determined, assuming that the bundle could be considered truly cylindrical. The fibre was then treated with solutions of hydrochloric acid of decreasing p_H, similar measurements of length and width being taken. The fibre-bundle was passed successively through the various solutions and was then thoroughly washed in distilled water. This same fibre was then treated in the same way and again washed and used a third time. The results are plotted in Fig. 1, which shows the influence of the p_H of the solution on the volume of the fibre-bundle. (The units of volume used in the figures are arbitrary micrometer units.)

The figure shows that the maximum swelling of a fibre-bundle of pure collagen occurs at p_H 1·8–1·9. It will be seen that taking the fibre through the

series of liquors up to p_H 0·3 brings about a change in the fibre-bundle, in that the apparent volume, when it is washed in distilled water after being used the first time, is greater than the original volume. After using twice, however, the apparent volume at p_H 5·0 is less than the original volume. This apparent decrease in volume is due to the breaking down of the fibre-structure, the fibre-bundle having a frayed appearance, and it is difficult to measure with any assured accuracy the exact width of the fibre. Owing to this fraying of the

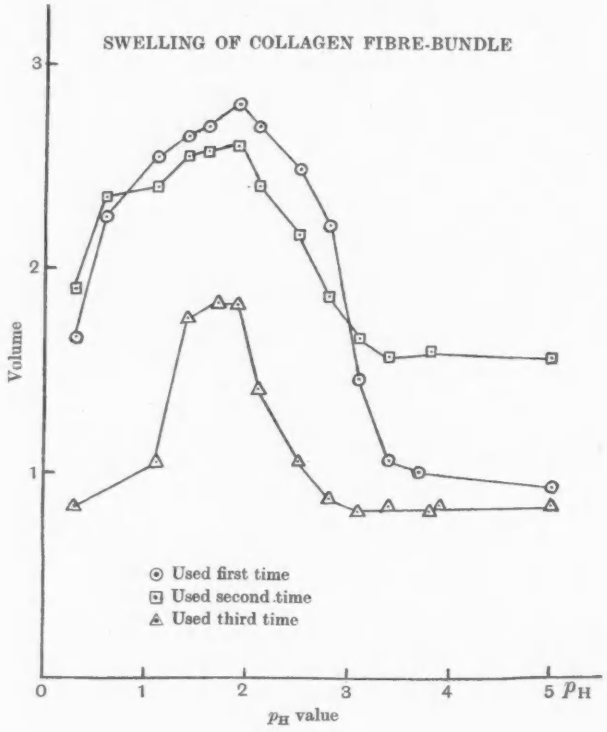

Fig. 1.

fibrils, it was not possible to use the fibre-bundle more than three times. The increased volume of the fibre at p_H 3·1–5·0, when used the second time, can be interpreted as due to the "opening up" of the fibre-bundle, *i.e.* the loosening of the finer elements of the fibre.

The curves show that increase in swelling only begins at p_H about 3·5 or less. It will be shown that where a marked increase in volume occurs in the range p_H 5·0–3·5, that this increase is not due to "plumping" but to "opening up." The more a fibre has been used, the more nearly horizontal is the part of the

curve between these p_H values. This means that opening up is a permanent change and that when it is complete the state so brought about is independent of p_H, at any rate (in the case of pure collagen) immediately on the acid side of p_H 5·0 and when hydrochloric acid is used to alter the p_H.

The curves in the region more acid than p_H 1·8 show a point of inflection at about p_H 1·1, when the fibre is used for the second and third times. In some cases this change in slope is found when the fibre is used for the first time, but when this occurs the point of inflection is generally at a slightly higher p_H. A careful examination of the graph shows that there is a slight indication of change of slope in the swelling curve of the fibre when used the first time, the point of inflection being at about p_H 1·6. This break in the curve was noted in the previous work of Kaye and Jordan-Lloyd [1924] and was found to be due to a structural change (splitting or opening up) of the fibre-bundle. It appears possible, however, that this type of opening up is rather different from that which can occur at $p_H > 3·0$. It also appears that the more the fibre-bundle is broken down by continued use, the less sharply defined is the point of maximum swelling. The general characteristic of the fibre-bundles appears to be that they become less and less sensitive to the action of reagents.

In the later work it was decided to abandon the method of direct measurement of the fibre-bundles, owing to its very tedious character, and to take photographs of the fibre-bundles at a known magnification and then to measure the photograph for length and to deduce its area by weighing the carefully cut out picture of the fibre-bundle. From these two measurements, the average width of the fibre-bundle could be computed and the volume calculated on the assumption that a cylinder, of which the length was that of the bundle and the diameter was that of the average width of the fibre-bundle, was equivalent in volume to the fibre-bundle.

The effect of the p_H on the swelling of a fresh hide fibre-bundle is shown in Fig. 2. This curve differs in several details from that shown in Fig. 1. There is a very marked increase in volume in the region p_H 3·5–5·0. This increase is due to the opening up of the bundle. The maximum swelling occurs at about p_H 2·8 and not at p_H 1·9 as in the pure collagen fibres. The significance of this difference will be discussed later.

The point of inflection in the region p_H 1·0 to 2·0 is at about p_H 1·6, whilst there is another break in the curve at about p_H 1·0. The significance of these two points is not yet known.

This method of expressing the results does not, however, throw light on the different ways in which the fibre can hold water. It may be convenient here to note that increase in volume may be brought about by three distinct processes. It can be caused by (*a*) an osmotic effect, (*b*) hydration of the protein, by which the water is bound to the protein bringing about an increase in bulk, (*c*) a physical separation of the component parts of the fibre-bundle. This last type of separation can be brought about by mechanical means, such as constant flexing or by alternately swelling and de-swelling the fibre. It is also possible

that the hydration of the protein leads to a separation of the individual structural units from each other. At the present stage of this investigation, however, it is not possible to distinguish between (*b*) and (*c*) and these two effects, which are probably closely inter-related, will be classed under the heading of "opening up." The osmotic uptake of water (*a*) has quite a different effect, and the difference between it and "opening up" can be clearly shown from the results already obtained.

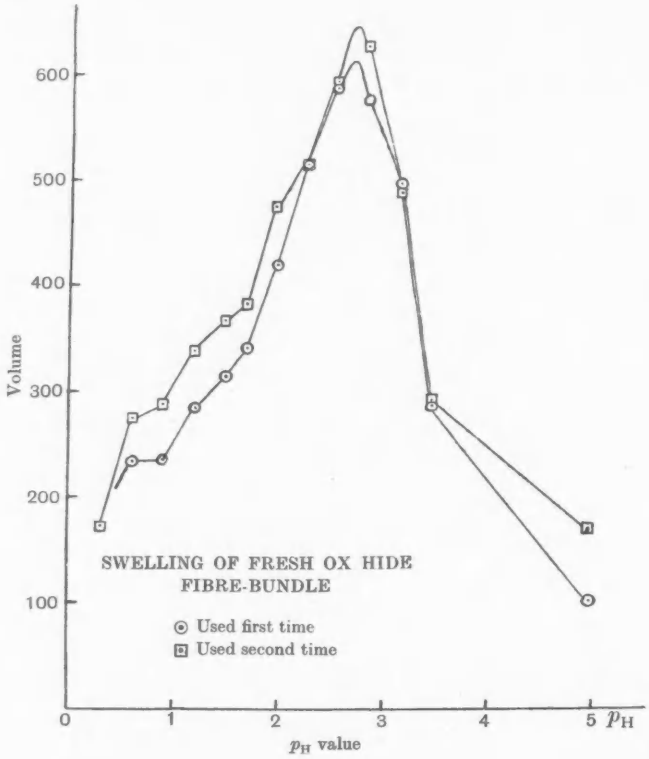

Fig. 2.

It was mentioned above that increasing the load on a fibre-bundle prevented the violent contortion which is normally produced by the action of $N/100$ hydrochloric acid, and that the constrictions in a swollen fibre caused by the encircling bands of reticular tissue could be made to disappear. Thus a fibre which was stretched could not "plump." This infers, therefore, that "plumping" is characterised by a contraction in length and an increase in thickness. Previous microscopical work has indicated that "opening up" is characterised

by an increase in thickness *only*. There is no apparent contraction in length. If these two dimensions of the fibre-bundle are plotted separately, it would be expected, therefore, that the decrease in length would be paralleled by an increase in width (or thickness) when the fibre-bundle was "plumped" but if "opening up" were taking place as well, the width would increase and would reach its maximum at a point different from that of minimum length. These effects are shown in Figs. 3 and 4, in which the alterations of length and breadth

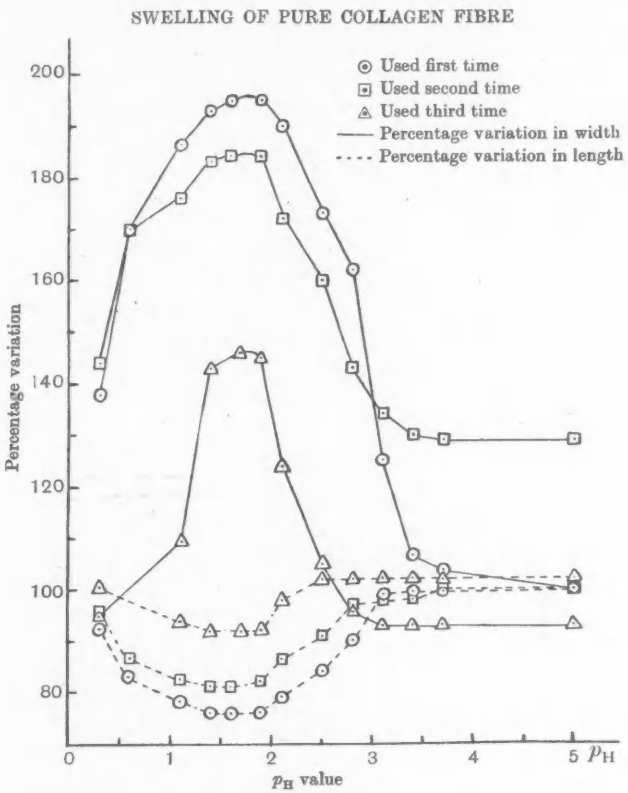

Fig. 3.

are expressed as percentages of the original dimensions of the fibre-bundle when in equilibrium with distilled water and are plotted against the p_H of the solution.

Fig. 3 shows the behaviour of a fibre-bundle of pure collagen. It will be seen that the first time the fibre is used the length does not begin to decrease appreciably until the p_H of the solution is less than 3·1. The increase in width, however, takes place at a slightly higher value (p_H 3·4). This suggests that some

"opening up" is taking place although its amount is not very great. The remaining portions of the two curves are almost inverse pictures of each other, the position of the maximum width occurring at about the same p_H (1·75) as the minimum length. When the fibre is used a second time, the width is appreciably greater than the original width because of the irreversible structural changes brought about in the first set of liquors. The increase in width with decrease in p_H begins more slowly than when the fibre is first used, but in this

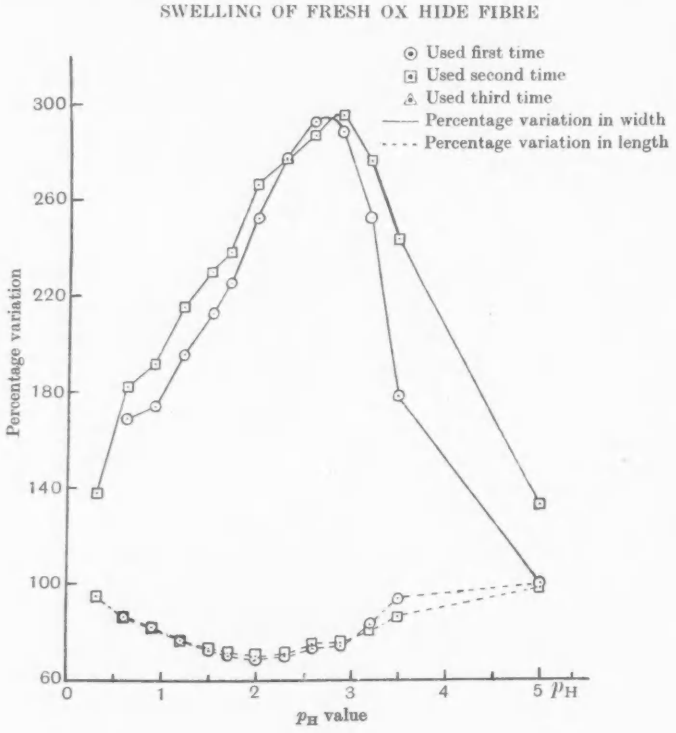

Fig. 4.

case it is accompanied by a definite, though slight, decrease in length. This suggests that the volume increase is more of the nature of "plumping." When the fibre is used the third time, the width does not increase until about p_H 3·0 is reached and no alteration occurs in the length until the acidity is greater than p_H 2·5, which may be interpreted as an indication of a small amount of "opening up."

Fig. 4 shows the results obtained by using a fresh ox hide fibre-bundle, and, apart from the greater amount of swelling, they differ considerably from those obtained when a fibre-bundle of pure collagen is used.

The increase in width is extremely rapid between p_H 5·0 and 3·5 and it is accompanied by only a comparatively small decrease in length. The decrease in length becomes marked at $p_H < 3·5$ and it is accompanied by a further increase in width. Thus, in this region (p_H 5·0–3·5) there is initially a large amount of "opening up" with a small amount of "plumping," and it is followed by a region in which "plumping" is probably the dominant factor. This region extends from p_H 3·5 to 2·9. At about p_H 2·8, the width reaches its maximum and there is a change in the rate of decrease in length. (This break in the curve which is similar to that which occurs in the case of pure collagen fibres, takes place, however, at a slightly different point, the p_H being 2·3–2·9 in the case of fresh hide and 1·9–2·5 for pure collagen.) The width of the fibre decreases as the p_H decreases from 2·7 to 2·0, but this decrease is accompanied by a further decrease in length. That is to say, the width is decreasing although the "plumping" is increasing. The decrease in width is caused, therefore, by a decrease in the "opening up" and not by a decrease in "plumping." The maximum "plumping" occurs at p_H 2·0, a value which is not very dissimilar from that for pure collagen.

The remaining portions of the curves, *i.e.* in the region more acid than p_H 2·0, have certain features similar to those found in the case of pure collagen, especially in the region p_H 0·6–1·2. In addition, there appears to be a break in the curve between p_H 1·2 and 2·0, but until further work has been carried out, it is not possible to interpret the significance of this.

Summary.

A microscopical examination of collagen fibre-bundles has been made using a method whereby the fibre-bundle is held so that accurate measurements can be made. By this technique it is shown that repeated swelling and de-swelling of the fibre-bundle causes it to become less sensitive to the swelling action of dilute acid solutions. It is also shown that the two factors, "plumping" and "opening up" can be distinguished one from the other and can be expressed in a general way by means of graphs. The point of maximum swelling of a fibre-bundle is that point where the sum of the swelling due to "plumping" and that due to "opening up" is greatest. This point is moved to a slightly higher value of p_H in the case of a fibre-bundle from fresh hide, where the amount of "opening up" is great, since the maximum "opening up" probably occurs at about p_H 3·0 to 3·5.

The author wishes here to express his thanks to the Council of the British Leather Manufacturers' Research Association for permission to publish these results.

REFERENCE.

Kaye and Jordan-Lloyd [1924]. *Proc. Roy. Soc. Lond.* B **96**, 293.

VI. INORGANIC CONSTITUENTS OF CEREBROSPINAL FLUID.

II. THE ULTRAFILTRATION OF CALCIUM AND MAGNESIUM FROM HUMAN SERA.

By ELSIE WATCHORN and ROBERT ALEXANDER McCANCE.

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(*Received December 21st, 1931.*)

In a recent paper McCance and Watchorn [1930–31] reported a preliminary investigation of the ultrafiltration of magnesium from serum. A further sixty cases have now been examined, including those with normal, subnormal and increased levels of serum-magnesium. No attempt has been made to study specifically any particular disease, as the main object has been to investigate the ultrafiltration of magnesium over a wide range of naturally occurring serum values. It is therefore impossible to deal statistically with separate diseases although some reference will be made to one or two, particularly nephritis. Normal pregnancy also will be discussed. As in the preliminary paper, calcium has been determined simultaneously in most cases for comparative purposes, although ultrafiltration of calcium from serum has already been extensively studied [for references see McCance and Watchorn, 1930–31].

Sources of material and technique.

The "normal" subjects were students, doctors or nurses, and were all in good health, leading active lives at the time the samples of blood were taken. All the other specimens of blood were taken from in- or out-patients of King's College Hospital. One of us [Watchorn, 1929] has shown that there is no appreciable variation in calcium and magnesium throughout the day, provided that violent exercise is not taken; consequently it is not necessary to ensure that the subject is fasting when blood is taken.

Experimental technique has been practically the same as that previously described [McCance and Watchorn, 1930–31]. To show that by this technique the ultrafiltrable calcium and magnesium can be accurately and consistently measured, a series of eight consecutive determinations is given in Table I. These were made on pooled samples of sera and each ultrafiltration was made with a separate membrane. It will be seen that the average deviations of the serum determinations are less than 1 %, and of the ultrafiltrates less than 2 %.

With regard to the ratio ultrafiltrable/total Mg or Ca it will be evident that the maximum possible variation for the series will extend from the lowest ultrafiltrable/highest total serum value to the highest ultrafiltrable/lowest total serum value. For magnesium this range is 0·732 to 0·779, and for calcium 0·533 to 0·561, indicating a possible maximum error of 6 % for the former and 5 % for the latter. The probable error is obviously less.

Table I. *Results on pooled sera.*

	Magnesium (mg./100 cc.)		Calcium (mg./100 cc.)	
	Serum	Ultrafiltrate	Serum	Ultrafiltrate
	2·65	2·01	10·29	5·68
	2·65	1·99	10·49	5·78
	2·62	2·04	10·29	5·59
	2·62	2·01	10·29	5·78
	2·65	2·07	10·29	5·78
	2·69	2·04	10·29	5·68
	2·65	1·97	10·39	5·78
	2·66	1·97	10·29	5·59
Mean	2·65 ± 0·014	2·01 ± 0·027	10·33 ± 0·06	5·71 ± 0·07

It is evident that the individual variations recorded have a real meaning and are not merely technical in origin. The results therefore can be dealt with collectively and, when occasion demands, individually.

Experimental results.

Table II contains the results obtained from eight normal persons; it will be seen that their calcium, magnesium and protein values conform closely to

Table II. *Normal subjects.*

		Magnesium mg./100 cc.			Calcium mg./100 cc.			Protein %	
Case No.	Sex	Serum (S.)	Ultrafiltrate (U.)	U./S. ratio	Serum (S.)	Ultrafiltrate (U.)	U./S. ratio	Total	Albumin
1	F.	2·45	1·73	0·706	—	—	—	7·50	—
2	F.	2·47	1·83	0·741	10·58	4·80	0·454	6·00	4·80
3	F.	2·49	1·88	0·755	10·29	5·10	0·496	7·70	5·10
4	F.	2·50	1·60	0·640	—	—	—	7·90	5·35
5	M.	2·42	1·85	0·764	10·88	5·40	0·496	7·35	4·70
6	M.	2·48	1·90	0·766	10·39	4·85	0·467	7·70	5·40
7	M.	2·48	1·85	0·746	9·99	5·40	0·541	6·65	4·95
8	M.	2·59	1·78	0·687	9·80	5·84	0·596	6·70	—

the generally accepted normal values. Table III contains all the other results arranged in ascending order of the serum-magnesium, grouped as follows.

(*a*) Values below 2·3 mg./100 cc., *i.e.* definitely subnormal.

(*b*) Values over 2·3 and below 2·66 mg./100 cc., *i.e.* covering the widest possible limits of normality.

Table III. *Pathological cases.*

Case No.	Diagnosis	Magnesium mg./100 cc. Serum	Magnesium mg./100 cc. Ultra-filtrate	Magnesium U./S. ratio	Calcium mg./100 cc. Serum	Calcium mg./100 cc. Ultra-filtrate	Calcium U./S. ratio	Protein % Total	Protein % Albumin
	(*a*) Serum values under 2·30 mg. Mg/100 cc.								
9	Uraemia	1·57	1·29	0·822	6·86	4·51	0·657	7·70	—
10	Pregnancy	1·97	1·57	0·797	9·11	4·80	0·527	6·80	4·95
11	Enlarged prostate	2·01	1·82	0·905	—	—	—	8·50	4·45
12	Pregnancy	2·09	1·68	0·804	9·31	4·80	0·516	5·25	3·65
13	,,	2·10	1·70	0·809	9·19	6·62	0·720	7·35	4·70
14	,,	2·17	1·82	0·839	9·02	5·59	0·620	5·60	3·74
15	,,	2·19	1·61	0·735	8·59	6·12	0·712	7·00	3·40
16	Before overbreathing	2·20	1·81	0·823	10·29	4·90	0·476	6·31	4·45
17	Hyperthyroidism	2·21	1·45	0·656	9·90	5·54	0·560	7·00	3·80
18	Enlarged prostate	2·24	1·87	0·835	12·08	5·54	0·459	6·30	—
19	Pregnancy	2·25	1·66	0·740	7·74	5·19	0·671	6·30	3·95
20	,,	2·26	1·68	0·743	8·50	5·33	0·627	6·30	—
21	After overbreathing	2·28	1·44	0·632	11·46	6·62	0·578	7·55	5·20
	(*b*) Serum values 2·30–2·65 mg. Mg/100 cc.								
22	Before overbreathing	2·34	1·57	0·671	10·96	5·64	0·514	—	—
23	Chronic parenchymatous nephritis	2·36	1·77	0·750	8·61	5·64	0·655	4·20	2·80
24	Pregnancy	2·47	2·11	0·854	8·92	6·17	0·692	6·15	4·20
25	,,	2·49	2·00	0·803	9·58	5·93	0·618	8·50	4·90
26	Lymphadenoma	2·51	1·91	0·761	9·26	6·06	0·654	4·20	3·50
27	After parathyroid therapy	2·52	1·81	0·718	10·59	4·85	0·458	—	—
28	Pregnancy	2·54	1·93	0·760	8·00	5·83	0·729	6·30	—
29	Diabetes	2·54	2·00	0·787	12·60	8·13	0·645	7·00	4·00
30	?	2·63	1·85	0·720	9·90	5·30	0·535	7·00	—
31	Pulmonary T.B.	2·64	1·79	0·678	10·64	5·00	0·470	5·97	4·65
	(*c*) Serum values 2·66–3·00 mg. Mg/100 cc.								
32	Acute anterior poliomyelitis	2·66	1·84	0·692	9·89	5·64	0·570	7·00	4·45
33	Pregnancy	2·70	2·04	0·756	9·58	6·81	0·711	8·05	5·80
34	After parathyroid therapy	2·70	1·87	0·693	9·40	5·00	0·532	7·50	—
35	Chronic parenchymatous nephritis	2·71	2·48	0·915	9·70	4·21	0·434	4·00	2·50
36	Enlarged prostate	2·72	2·04	0·750	12·45	7·55	0·606	7·15	4·45
37	Eclampsia	2·73	1·98	0·725	9·09	5·33	0·485	6·30	3·40
38	Nephritis	2·73	2·02	0·740	10·99	4·26	0·469	7·00	—
39	Uraemia	2·77	1·92	0·693	7·12	5·33	0·748	6·50	4·80
40	After overbreathing	2·77	1·74	0·628	10·49	5·19	0·495	—	—
41	Asthma	2·79	2·24	0·803	11·17	6·17	0·553	—	—
42	Lead neuritis	2·81	1·99	0·708	9·70	5·90	0·608	6·90	—
43	Uraemia	2·86	1·88	0·657	12·24	5·74	0·469	7·45	5·10
44	Chronic nephritis	2·91	2·51	0·862	9·58	5·83	0·609	7·30	4·30
45	Pregnancy and nephritis	2·96	2·70	0·912	9·70	5·70	0·588	5·70	—
46	Asthma	3·00	1·94	0·647	9·31	5·78	0·621	—	—
	(*d*) Serum values over 3·00 mg. Mg/100 cc.								
47	Carcinoma of oesophagus	3·07	1·95	0·635	11·16	6·62	0·593	6·30	4·00
48	Asthma	3·14	2·38	0·758	11·56	5·98	0·517	—	—
49	Pulmonary T.B.	3·17	2·36	0·745	7·11	4·74	0·667	5·80	—
50	Streptococcal infection of throat	3·18	2·14	0·673	12·67	6·28	0·496	7·90	5·35
51	Uraemia	3·26	2·62	0·804	6·42	4·84	0·754	8·50	3·50
52	After intramuscular injection of magnesium	3·33	2·14	0·643	—	—	—	—	—
53	Nephritis	3·43	2·50	0·729	10·57	7·01	0·663	7·15	4·80
54	After intramuscular injection of magnesium	3·53	3·00	0·853	10·98	6·70	0·610	—	—
55	Diabetic coma	3·70	2·70	0·730	13·00	8·13	0·625	8·10	4·00
56	T.B. meningitis	3·81	3·38	0·887	12·03	6·32	0·525	—	—
57	Uraemia	3·92	3·28	0·837	3·38	2·06	0·609	6·20	3·80
58	,,	3·95	2·88	0·729	8·81	5·01	0·569	6·30	4·65
59	Uraemia caused by chronic lead poisoning	5·77	4·27	0·740	13·62	7·98	0·586	6·95	5·35
60	Uraemia	10·34	7·86	0·760	3·82	2·35	0·615	6·30	4·30

(*c*) Values over 2·65 and below 3·00 mg./100 cc., *i.e.* definitely above normal. This class includes the great majority of hospital patients [McCance and Watchorn, 1930–31].

(*d*) Values over 3·00 mg./100 cc., *i.e.* wide departure from normal.

Table IV contains the averages of the normals and the four pathological groups, and also the averages within each group of the ratio of the ultrafiltrable magnesium to the total serum-magnesium (U./S. ratio). In this Table the results are also expressed as millimols per litre, correction having been made in the case of serum for the space occupied by proteins, according to the formula:

$$\frac{M \times 100}{(100 - P)}$$

where M represents millimols per litre of Mg or Ca, and P the percentage of total serum-proteins.

Table IV. *Average values.*

(I) Magnesium.

Range (mg./100 cc.)	Serum (S.) mg./100 cc.	Ultra-filtrate (U.) mg./100 cc.	U./S. ratio	Serum liquid (S.L.) millimols per litre	Ultra-filtrate millimols per litre	U./S.L. ratio	Serum-protein %
Normals	2·48	1·80	0·726	1·10	0·74	0·673	7·19
Group *a*. 1·57–2·28	2·11	1·65	0·782	0·93	0·68	0·731	6·77
„ *b*. 2·34–2·64	2·50	1·87	0·748	1·10	0·79	0·718	6·18
„ *c*. 2·66–3·00	2·79	2·08	0·745	1·22	0·86	0·705	6·48
„ *d*. over 3·00	3·63	2·74	0·751	1·64	1·13	0·689	7·02
Average for whole series	2·74	2·03	0·750	1·17	0·83	0·709	6·72

(II) Calcium.

Normals	10·32	5·23	0·508	2·77	1·28	0·462
Group *a*	9·34	5·46	0·584	2·50	1·36	0·544
„ *b*	9·91	5·85	0·590	2·64	1·46	0·553
„ *c*	10·03	5·63	0·561	2·68	1·41	0·526
„ *d*	9·62	5·69	0·592	2·55	1·42	0·557
Average for whole series	9·79	5·61	0·573	2·62	1·40	0·534

Discussion.

The relationship of the ultrafiltrable to the total serum-magnesium and calcium.

Table IV shows that the average ratio of the ultrafiltrable/total serum-magnesium for the whole series is 0·750, and that the average ratios of the normal group and of Groups *a*, *b*, *c* and *d*, are all very close to this figure. The lowest individual figure is 0·628 and the highest 0·915, while the average deviation for the whole series is 0·058. It is evident therefore that however much magnesium there is in the serum in general the same fraction of it is ultrafiltrable, and that even individual variations are not very great. When corrections are made for the space occupied by the serum-proteins the ratio for the whole series (ultrafiltrable Mg/Mg in serum liquid) is 0·709.

The average ultrafiltrable calcium for the whole series is 5·61 mg./100 cc., or, expressed as a ratio of the total serum-calcium, 0·573. This is higher than Greenberg and Gunther's average [1930], and higher than the average ratio of our normal subjects, which is 0·508. As with magnesium the ultrafiltrable calcium tends to vary directly with the total serum-calcium. Thus, for all the serum-calcium values under 10 mg./100 cc., the average ultrafiltrable calcium was 4·97 mg./100 cc., and for serum-calcium values over 10 mg./100 cc. the ultrafiltrable value was 6·03 mg./100 cc. The corresponding ratios are 0·617 and 0·532 respectively. Case No. 60 is a good example of the constancy of the ultrafiltrable/total serum ratio (U./S. ratio) both for magnesium and calcium, even when the serum values are grossly abnormal. Thus, with a total serum-Mg of 10·34, the U./S. ratio is 0·760 and with a total serum-Ca of 3·82 the U./S. ratio is 0·615.

There is no relation between the level of calcium and of magnesium in the serum, both average values (Table IV) and individual cases show that these two blood constituents vary independently of each other.

It appears from these figures that on an average about 25 % of the total magnesium of serum and 41 % of the total calcium are non-diffusible. It is generally presumed that this non-diffusible calcium or magnesium is bound to the serum-proteins [Peters and Van Slyke, 1931, p. 811]. If this presumption is correct one would expect that the ratio of the ultrafiltrable/total serum-Mg or Ca would vary inversely with the total serum-proteins, for the smaller the protein content the more magnesium or calcium will be free to pass into the ultrafiltrate. When all the figures under review are considered together it is impossible to demonstrate this point. Taking all the available data from Tables II and III they can be arranged so that all cases with serum-proteins below 7 % form Group I and all with proteins 7 % or above Group II. The average U./S. ratios then obtained are:

Group I (serum-proteins below 7 %)	0·588 Ca U./S.	0·767 Mg U./S.
Group II (serum-proteins 7 % or above)	0·590 „	0·745 „

From these figures it appears that the amount of magnesium or calcium found in an ultrafiltrate is practically unaffected by the level of proteins in the serum. This is shown also in Table IV where considerable variations in serum-proteins are not accompanied by corresponding changes in the ultrafiltrable fraction of the serum-magnesium or -calcium. Many of these cases, however, though not showing marked abnormalities in the total serum-protein level have greatly disturbed relative amounts of albumin and globulin. When such cases are eliminated from the series (that is to say, those cases having albumin/globulin ratios outside the widest limits of normal, 1·6–4·0), then the relation between the serum-protein level and the ultrafiltrable Ca or Mg is:

Group I (serum-protein below 7 %)	0·581 Ca U./S.	0·767 Mg U./S.
Group II (serum-protein 7 % or above)	0·570 „	0·719 „

Again the lack of relation between the calcium and the serum-protein level is evident, but the results seem to indicate that in the majority of cases there is

a tendency for the ultrafiltrable magnesium to be inversely proportional to the total serum-protein. A further analysis of the figures confirms this:

Proteins below 6 %	magnesium U./S. ratio	0·797.
Proteins 6–7 %	„ „	0·752.
Proteins 7 % or over	„ „	0·718.

The fact that this relation is not universal over the whole range of cases under consideration suggests that some other factor besides the amount of protein present in the serum affects the ultrafiltrable magnesium, and may in many instances be the chief agent affecting the non-diffusible fraction. If the serum-magnesium and -calcium in colloidal form are not entirely in direct combination with the proteins they may be in part in combination with the organic lipoid phosphoric acids of the serum. The salts of these acids would resemble alkali protein salts in behaviour. These acids have the requisite properties therefore and are present in sufficient quantity to bind considerable amounts of Mg and Ca in colloidal form. Presumably also these elements can vary independently of the proteins [Peters and Van Slyke, 1931, p. 1102]. Both Brull [1928] and Scholtz [1931, 2] believe that calcium and magnesium form colloidal phosphate complexes. This point is being further studied.

Though most writers have suggested that the serum-calcium level is influenced by the percentage of protein present, Stearns and Knowlton [1931] failed to find any relation between these two constituents in the sera of normal children.

Pregnancy.

Twelve cases were examined between the 32nd and 38th week of gestation. Their Case Numbers are 10, 12, 13, 14, 15, 19, 20, 24, 25, 28, 33, 45. Four points require discussion. (*a*) The total proteins in the serum average 6·60 % which is below the generally accepted normal value of 7 % [Salvesen, 1926] and below the normal average of 7·19 % found in the present study. This reduction of serum-protein during pregnancy is a recognised phenomenon. According to Plass and Bogert, Coetzee, and Oard and Peters [quoted from Peters and Van Slyke, 1931, p. 663] the serum-proteins are at their lowest level (average 6·2 %) at the sixth month of pregnancy and thereafter rise to nearly normal at term. The average of 6·6 % therefore at the 7–8th month agrees well with these observers. Kydd [1931] found that in pregnancy the serum-proteins were reduced at the expense of the albumin fraction. The present results tend to confirm this also. The albumin/globulin ratio of the small normal series is 2·3 while that of the pregnant series is 1·93. (*b*) The low serum-calcium found by many other workers [*e.g.* Aburel and Ornstein, 1930; Bokelmann and Bock, 1928; Plass and Bogert, 1923; Spiegler and Schol, 1929; Widdows, 1923] is here shown again. All the values are below 10 mg./100 cc. and 5 are below 9 mg./100 cc. (*c*) In 7 cases the serum-magnesium is also definitely low, in 3 it is normal and in 2 raised. One of the last was complicated by nephritis and therefore should probably be excluded from consideration here. As it is

unusual to find a serum-magnesium below normal, most known pathological processes tending to give a high figure, it is evident that pregnancy tends to lower the serum-magnesium as well as the serum-calcium. This generalisation cannot be too strictly applied to the individual cases where, for example, a low magnesium may be associated with a normal calcium and *vice versa*. (*d*) The ultrafiltrable fraction of the total serum-magnesium was increased. The average ultrafiltrable/total Mg (U./S.) ratio is 0·796, compared with a ratio of 0·726 for the normal cases, and 0·750 for the whole series. The ultrafiltrable fraction of the calcium is also increased, the average ratio being 0·643, against a ratio of 0·508 for the small normal series, and 0·573 for the whole series. Although the number of pregnant cases is rather small (12), and the results must be confirmed on a larger series suitably controlled, they suggest that during pregnancy a larger amount of the magnesium and calcium is ultrafiltrable than in normal conditions. This is not satisfactorily explained by the diminished quantities of protein in the serum during pregnancy, because (i) the massed results of the present study have failed to demonstrate any such dependence (*v. supra*); (ii) individual cases, for instance Nos. 13 and 25, may show a high proportion of ultrafiltrable Mg or Ca associated with a high percentage of serum-protein. Factors of which we have no certain knowledge are evidently at work in these cases. We can only say that some change in the normal physico-chemical equilibria must have taken place. Whatever the underlying mechanism, the increasing readiness with which calcium and magnesium can be ultrafiltered from the serum during pregnancy may be of considerable biological significance, because everything that passes through the placenta must presumably be in ultrafiltrable form. The physico-chemical changes that take place in the blood during pregnancy therefore favour the passage of these two essential elements from mother to foetus.

Uraemia and nephritis.

Eight cases are available for discussion, Nos. 9, 39, 43, 51, 57, 58, 59, 60. No. 59 was the result of chronic lead poisoning. These patients all showed a high degree of N-retention. Most had blood-ureas of 200–300 mg./100 cc. and two of over 800 mg./100 cc. (Nos. 51 and 57). Remarkable variations of both serum-calcium and -magnesium have been found in this group, magnesium values ranging from 1·57 to 10·34 mg./100 cc. and calcium values from 3·38 to 12·24 mg./100 cc. These highly abnormal fluctuations have not been accompanied by corresponding protein changes and even the extreme cases have shown normal U./S. ratios both for calcium and magnesium. Whatever their meaning these results do seem consistent and definite. Salvesen and Linder [1923, 2] and Pincus, Peterson and Kramer [1926] have claimed that the low blood-calcium associated with a normal amount of filtrable calcium found by them in non-uraemic nephritis is the result of a decrease in serum-proteins, and that the absence of tetany in these cases can be thus explained. It is evident that in uraemia the low serum-calcium is not the result of lowered serum-

proteins or associated with raised U./S. ratios. Tetany has never been observed in any case in this series even when the serum-calcium was as low as 3·38 mg./100 cc.

Five cases of nephritis without uraemia have been studied (Nos. 23, 35, 38, 44 and 45). Nos. 38, 44 and 45 were of the mixed type so often seen clinically with some N-retention and some oedema. These require no comment. Nos. 23 and 35 were diagnosed clinically as chronic parenchymatous nephritis and merit further consideration. Both had extensive oedema, complete absence of N-retention and very low serum-proteins. According to Salvesen and Linder [1923, 1] and Pincus, Peterson and Kramer [1926] one would have expected to find in both a low serum-calcium and a high ultrafiltrable/total Ca ratio. On general grounds also one would have expected the ultrafiltrable magnesium to have run parallel with the ultrafiltrable calcium. The results actually obtained were:

	Serum-Ca mg./100 cc.	U./S. ratio	Serum-Mg mg./100 cc.	U./S. ratio
No. 23	8·61	0·655	2·36	0·750
No. 35	9·70	0·434	2·71	0·915
Normal	9–11	0·508	2·3–2·65	0·726

It will be noted that (*a*) the predicted relationship holds for the Ca of No. 23, (*b*) it cannot be said to apply to the Mg of the same case, (*c*) the serum-Ca of No. 35 is strictly normal and the U./S. ratio low, (*d*) the serum-Mg is over normal and its U./S. ratio very high. These individual results, which cannot be explained away on technical grounds (see Table I), are quoted to show that low serum-proteins may be associated with abnormally high or low U./S. ratios, and that the U./S. ratio of Ca and Mg in the same serum may vary very widely from the normal in different directions. Many other examples of this may be found in the Tables. As one would expect any external change of conditions such as p_H to alter the ultrafiltrability of Ca and Mg in the same direction it is probable that the cause of these differences must lie in the serum itself.

Eclampsia.

The only case studied (No. 37) showed no abnormalities. There was no reduction of the ultrafiltrable calcium such as Rodecurt, Koenig and Regensburger [1928] found, and no reduction of the total calcium such as Halverson, Mohler and Bergeim [1917] reported.

Parathyroid administration.

Only one case has been available for study. This was a man aged 59 suffering from peripheral neuritis due to lead poisoning (case Nos. 42, 34 and 27). Collip's parathormone was given intramuscularly.

During treatment the total serum-Mg fell definitely but slightly and with it the ultrafiltrable Mg, so that the ratio U./S. remained constant. The variations in the latter are within the limits of experimental error. The total

serum-Ca did not rise beyond normal limits. Hunter and Aub [1926–27] have reported that in similar cases parathyroid sometimes failed to raise the serum-calcium, even when given in massive doses. In the case under discussion there was a progressive and definite fall in the ultrafiltrable fraction of calcium, which is in harmony with the findings of Cameron and Moorhouse [1925]; Cruickshank [1923, 1, 2] and Merritt and Bauer [1931], but contrary to those

	Magnesium			Calcium		
	Serum	Ultra-filtrate	U./S. ratio	Serum	Ultra-filtrate	U./S. ratio
Before treatment	2·81	1·99	0·708	9·70	5·90	0·608
After 6 days during which 180 units parathormone were given	2·70	1·87	0·693	9·40	5·00	0·532
After 6 more days during which 375 units parathormone were given, *i.e.* 555 units in 12 days	2·52	1·81	0·728	10·59	4·85	0·458

of Liu [1927], and of Snell and Wales [1930]. Moritz [1925] and Reed [1928], who also investigated the effect of parathormone on the ultrafiltrable or colloidal calcium in serum, have obtained inconclusive results. Scholtz [1931, 1] found that parathormone raised the serum-magnesium for a few hours after the injection, but his investigation is not comparable with ours, for which blood was never withdrawn within 6 hours of an injection of parathormone.

The partition of magnesium and calcium between the serum and cerebrospinal fluid.

Stary, Kral and Winternitz [1929] and McCance and Watchorn [1930–31] have each concluded that there is no relation between the diffusible serum-magnesium and the cerebrospinal fluid-magnesium. The present study confirms this opinion. In the whole range of 60 cases here presented the percentage of magnesium found in the ultrafiltrate only rises as high as the average normal C.S.F. percentage in Nos. 56, 59 and 60. Further the amount of magnesium found in an ultrafiltrate depends mainly upon the amount originally present in the serum, whereas the C.S.F.-magnesium tends to be constant in amount and independent of the serum value [McCance and Watchorn, 1930–31]. Case No. 60 illustrates this. The serum-magnesium was grossly abnormal (10·34 mg./100 cc.), the ultrafiltrate contained 7·86 mg./100 cc. and the ratio of the two was 0·760, which is normal. Yet the C.S.F. of this case contained only 4·69 mg./100 cc., which although definitely above normal was not much more than half the concentration in the ultrafiltrate.

Opinion is still divided as to whether ultrafiltration will wholly explain the calcium found in the cerebrospinal fluid. There is no doubt that when a series is averaged the C.S.F.-calcium and the ultrafiltrable calcium lie very close

together, and that both tend to rise and fall with the total serum value. In individual cases however the C.S.F. and ultrafiltrate values do not agree and the differences cannot be dismissed as experimental errors [see McCance and Watchorn, 1930–31, Tables III and IV]. Morgulis and Perley [1930] state that the C.S.F.-calcium is relatively much more constant in amount than the serum-calcium, and only follows to a slight degree experimental variations in the latter. Brucke [1925] found the C.S.F.-calcium relatively independent of that of the serum. Moreover, although Critchley and O'Flynn [1924] found the C.S.F.-calcium very much reduced in tetany, Leicher [1922–23] has reported a case of tetany with a very low serum-calcium and a normal C.S.F.-calcium. Hertz [1930] also, working with dogs, came to the conclusion that in acute variations of the serum-calcium the ultrafiltrable calcium no longer corresponds with the C.S.F.-calcium. One case from the present series (No. 60) shows definitely that the C.S.F.-calcium may be entirely dissociated from the ultrafiltrable calcium of the corresponding serum. The serum-calcium of this case was only 3·8 mg./100 cc.; the ultrafiltrable calcium was 2·35 mg./100 c.c., so that the U./S. ratio (0·615) was nearly normal; yet the C.S.F.-calcium was 4·21 mg./100 cc., practically in the normal range and actually higher than the serum-calcium. It is possible therefore that agreement between the ultrafiltrable and C.S.F.-calcium over the normal range of serum values is merely fortuitous and that the C.S.F.-calcium is regulated at its level of 4–6 mg./100 cc. by some mechanism allied to that which maintains the C.S.F.-magnesium about 3·33 mg./100 cc. From this it is evident that the opinion of Merritt and Bauer [1931] that the cerebrospinal fluid-calcium content is "a more accurate physiological representation of the diffusible calcium of the serum than the values obtained by the various filtration methods" cannot be entirely accepted.

Summary.

(1) Eight normal and 52 pathological cases have been examined.

(2) A very constant fraction (about 75 %) of the total serum-magnesium is ultrafiltrable. On an average 57 % of the serum-calcium is ultrafiltrable, so that a smaller proportion of the serum-magnesium than of the serum-calcium is present in colloidal form.

(3) The proportion of the total serum-calcium that is ultrafiltrable is not affected by the proteins in the serum. The ultrafiltrable magnesium may be inversely proportional to the total serum-protein within certain limits. This has been discussed. In the same serum the ultrafiltrable/total serum-calcium ratio may be very high and the magnesium ratio very low.

(4) In pregnancy the serum-magnesium as well as the serum-calcium tends to fall and a higher proportion of both tends to be ultrafiltrable.

(5) Great variations in both serum-magnesium and calcium may be encountered in the last stages of chronic interstitial nephritis (uraemia), but the ultrafiltrable/total ratio remains within normal limits.

(6) After parathormone administration one case showed a progressive fall in the ultrafiltrable/total serum-calcium ratio.

(7) The concentration of magnesium in the cerebrospinal fluid cannot be explained by ultrafiltration. Ultrafiltration may explain the C.S.F.-calcium over normal ranges of serum-calcium values although such an explanation is not wholly satisfactory. Ultrafiltration cannot explain the C.S.F.-calcium in some cases with very low serum-calcium values.

E. W. is indebted to the Medical Research Council for a whole time personal grant. R. A. McC. has carried on this work during the tenure of a Pinsent Darwin Studentship which he acknowledges with thanks.

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VII. THE EFFECT OF ALCOHOL ON THE OXYGEN UPTAKE OF BRAIN TISSUE.

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(*Received December 21st, 1931.*)

VARIOUS theories have been proposed to account for the symptoms which follow the ingestion of ethyl alcohol, but it seems to be generally agreed that the drug has a purely depressant action. The apparent stimulation which follows quickly on the taking of alcohol is ascribed to a depression of the higher centres with a weakening of inhibitory control. The spreading of this depression to other—and "lower"—centres either later or with larger doses accounts for the loss or partial loss of the control over the more automatic functions of speech and balance. It is not necessarily suggested that the drug acts on certain brain cells preferentially, but rather that, all cells being affected, the resulting functional deficiency is first shown by those responsible for the reflexes most recently acquired, and, therefore, most readily disturbed.

If this idea of the mechanism of alcoholic intoxication be accepted, the question at once arises as to how the alcohol exerts its depressant effect, and so a complete answer to this question may indeed be expected to afford evidence as to the correctness, or otherwise, of the hypothesis to which it is a corollary.

Ethyl alcohol, in common with the other members of the same chemical series, with the urethanes and with other narcotics, is powerfully adsorbed on such colloidal surfaces as constitute enzyme systems. This property is one of which great use has been made in elucidating the mechanism of enzyme action. *A priori* then, it seems possible that ethyl alcohol may owe part at least of its toxic properties to its adsorption on the oxidative enzyme surfaces of the brain cells, resulting in a partial suppression of the normal metabolic processes. Such a depression would never be complete, since it would depend on a competition for these surfaces between the alcohol and the normal metabolites, but it would obviously to some extent be proportional to the concentration of alcohol present, at any rate below some limiting value.

It appears then that a study of brain metabolism in the presence of ethyl alcohol might be expected to throw some light on the way in which the drug exerts its toxic action, and such a study we have commenced. As a first stage we have considered only the total metabolism as measured by the oxygen

uptake of the excised brain tissue, and it is these experiments, with the somewhat tentative interpretation we have placed upon them, which we wish to describe in this paper.

Experimental.

We thought it desirable that the alcohol should reach the brain cells in the normal way; that, as far as possible, inaccuracies due to diffusion into and from the tissue during the measurements of oxygen uptake should be avoided; and that as little time as possible should be lost between the extraction of the brain and the commencement of measurements. For these reasons we administered alcohol to living rabbits which were killed after a suitable time had elapsed. At first the drug was given by stomach tube, but this procedure was soon found to give very irregular results and was therefore discarded. It appeared that the large amount of food present in the stomach, even after 24 hours' starvation, hindered the absorption of alcohol. When however alcohol was given *per rectum*, it was more quickly and more completely absorbed.

The dose was 5 cc. of absolute alcohol per kg. body weight, diluted to three times its volume with distilled water. Obvious signs of intoxication usually appeared about 20 minutes after administration of alcohol; the animal gradually lost control of its limbs and in most cases finally passed into a comatose condition, from which, however, it apparently completely recovered, within 4 hours.

The animals were killed at times varying from 15 minutes to 4 hours after receiving alcohol, and, in addition, normal controls were examined. The following eight series of experiments were thus made: (*a*) controls (no alcohol); killed (*b*) 15 minutes, (*c*) 30 minutes, (*d*) 1 hour, (*e*) 1½ hours, (*f*) 2 hours, (*g*) 3 hours, (*h*) 4 hours, after alcohol.

The brain was quickly extracted without special aseptic precautions. With clean instruments, bacterial infection was a factor which could safely be ignored during the 3 hours which the observations lasted. The whole brain was placed for 20–25 minutes in the quick-cooling cabinet of a refrigerator. It had then assumed a cheese-like consistency and could readily be cut into thin, even slices with a razor. In each experiment we used (*a*) slices through the middle of the brain, consisting of both grey and white substances, but chiefly the latter, and (*b*) slices from the surface of the cortex which consisted chiefly of grey matter.

The weighed slices (two for each observation) were suspended in either phosphate-Ringer solution of p_H 7·4, or in the same solution *plus* 0·4 % (by volume) of alcohol. This latter solution corresponds to approximately 0·35 % of alcohol by weight and this, analysis showed, was the average concentration in the brain tissue under the conditions of our experiments. It seemed reasonable to suppose that the presence of alcohol in this concentration in the fluid surrounding the tissue would give a truer picture of the state of affairs *in vivo*, since the tissue suspended in buffer alone must lose alcohol from the surface

cells at least. In it, therefore, the concentration of alcohol would decrease for a while, so introducing an unknown (and, in view of the minute quantities involved, incalculable) factor into the oxygen uptake.

The oxygen uptake was measured in Barcroft differential manometers, using flat-bottomed cups of about 70 cc. capacity. The volume of fluid in each cup was 2 cc., and the control cup always contained fluid of the same composition as that containing the brain slices. The apparatus was carefully calibrated at the temperature of the thermostatically controlled water-bath (37°) by introducing known volumes of air from a standardised pipette. As usual, it was necessary to allow 10 minutes for equilibration of the manometers, but thereafter readings were taken at 10-minute intervals over a period of 3 hours. In some cases observations were continued for a fourth hour, but usually by this time the oxygen consumption rate was beginning to decrease markedly, owing presumably to the cells "dying." In a few instances however the oxygen uptake increased markedly during the fourth hour, and this was ascribed to bacterial contamination. In any case, these irregularities made the results obtained during this period practically valueless, and they were perforce discarded for the purposes of interpretation.

Corrections were applied to the readings for calibration, barometric pressure, water vapour pressure and temperature. The oxygen uptake was thus calculated for each 10-minute period in $mm.^3$ at N.T.P. per g. of tissue.

Results.

The mean value of the figures obtained for the oxygen uptake per g. of brain tissue in the control series was of the same order as that reported by Holmes and Ashford [1930], though the variation was rather greater. We had used slices, whereas those authors used chopped tissue. Similar variations from brain to brain were found in each series, in spite of the fact that conditions were kept as nearly constant as possible, while the experiments were necessarily regarded as satisfactory in that they indicated, for each sample, a regular uptake of oxygen, and duplicate samples showed moderately good agreement (within 10 %). It was impossible, however, to draw valid deductions from the comparison of individual results in different series, and it was necessary to consider only the mean values from each series.

These mean figures were, in the first instance, plotted in the usual way—oxygen uptake against time in the Barcroft manometer. As had been expected the experiments in which the brain was suspended in Ringer containing alcohol gave the most regular results. Curves obtained from these experiments are shown in Fig. 1. It can be seen from this figure that during the first hour of respiration *in vitro*, those brains from animals killed within an hour of receiving alcohol showed a markedly increased rate of oxygen usage as compared with non-alcoholised brains. When the animal was killed 1½ hours after alcohol the brain showed an approximately normal rate of oxygen uptake, but with

longer intervals between alcohol ingestion and death there was a tendency, maximal at about 3 hours, to a reduced rate of oxygen usage.

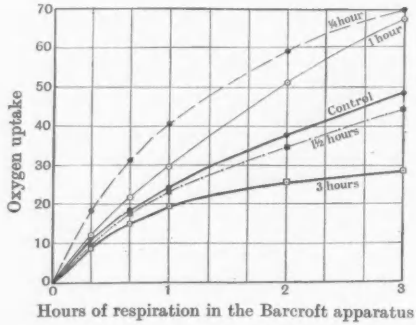

Fig. 1. Oxygen uptake of whole-brain tissue suspended in Ringer solution containing 0·4 % of alcohol. The curves are marked to show the time which elapsed between the administration of alcohol and the death of the animal.

During the third hour of respiration in the Barcroft apparatus, those tissues which had earlier shown an accelerated metabolic rate—*i.e.* those from animals killed within an hour of receiving alcohol—were using oxygen only at about the same rate as the controls. Those, which had earlier shown a depressed rate of oxygen usage, now showed a slight tendency to approach more nearly to the normal rate.

It seemed, therefore, that the initial effect of alcohol was to stimulate the consumption of oxygen, an effect lasting, in these experiments, for an hour, and that this was succeeded by a depression in oxygen usage.

In order to demonstrate these effects more clearly, and to allow of further examination as to their reality, the figures were analysed in another way. A single example will suffice to explain the method of charting, which is employed in Figs. 2–6. Thus in Fig. 2 we have taken from each series of experiments the mean figure for the oxygen uptake during the first 20 minutes in the Barcroft apparatus, and plotted it against the time which elapsed between the administration of alcohol and the death of the animal. This chart gives, most nearly, information regarding the oxygen usage in the brain at the time the animal was killed; the subsequent charts, which show the oxygen uptake after increasing survival periods, must be regarded, at any rate until further examination has shown their substantial accuracy, as giving progressively less reliable information. By measuring the oxygen uptake of the brain as rapidly as possible after death we obtain, as nearly as can be, a picture of what is happening at the time the animal is killed. By killing the animal at various times after the administration of alcohol we have obtained a series of such pictures. We have, therefore, a continuous record of the effect of alcohol during the first 4 hours of its action.

Fig. 2 emphasises the deductions drawn from Fig. 1, and extends them. In every case, whether the tissue consisted principally of white, or principally of grey matter, and whether the suspension fluid was phosphate-buffer alone or contained alcohol, there is an increased oxygen usage in those animals killed a quarter or half an hour after the administration of alcohol. The increase is greater in the tissue composed mainly of grey matter, and, with the grey matter suspended in Ringer, is still found in those animals killed 1 hour after administration of alcohol. In white matter, however, this stimulation has disappeared,

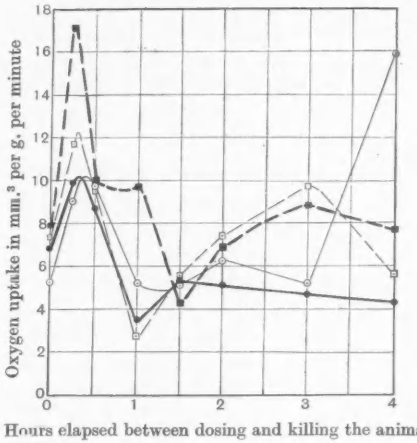

Fig. 2. The oxygen uptake per minute of brain tissue during the first twenty minutes of respiration in the Barcroft apparatus, plotted against the time during which alcohol was in contact with the brain *in vivo*.

—●—●— Whole brain in Ringer solution.
—⊙—⊙— Whole brain in alcoholic-Ringer.
—■—■— Grey matter in Ringer solution.
—⊡—⊡— Grey matter in alcoholic-Ringer.

or been replaced by a depression, in the animals killed 1 hour after receiving alcohol. Thereafter the oxygen uptake remains at nearly the normal value until the animal has been "alcoholised" *in vivo* for 4 hours. In the case of whole brain suspended in alcoholic-Ringer there is then a sudden, and at present inexplicable, jump to far above the normal value. The figure from this series, however, must be accepted with some caution, for though it is as usual the mean of a number of observations, and these observations were made at the same time as the corresponding ones for whole brain in Ringer (which shows no such abnormality), it is founded on a relatively small number of experiments.

The second 20 minutes of *in vitro* respiration (Fig. 3) shows an essentially similar picture, except that the early increase in oxygen uptake is less marked, and is, indeed, absent from the whole brain suspended in Ringer alone.

In the third 20 minutes (Fig. 4) only the grey matter shows an increase in the case of animals killed within a short time of receiving alcohol. This increase is smaller but still appreciable. Both Figs. 3 and 4 show again the curious increased metabolism in the animals killed 4 hours after receiving alcohol, but, as before, only for whole brain tissue suspended in alcoholic-Ringer.

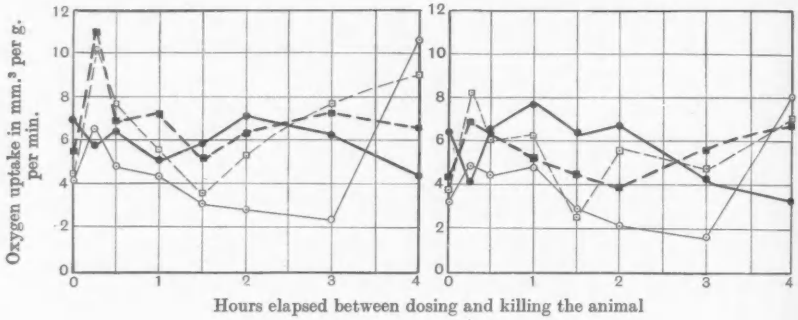

Fig. 3. Fig. 4.

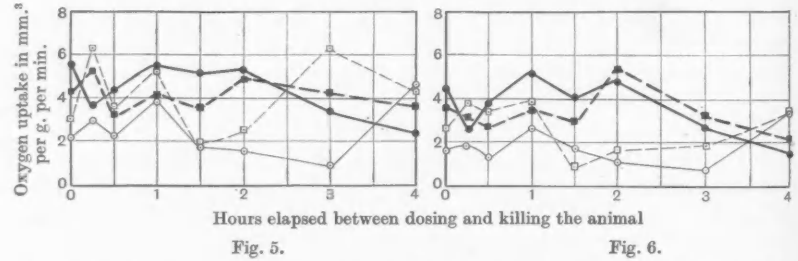

Fig. 5. Fig. 6.

Figs. 3–6. The oxygen uptake per minute of brain tissue during the second twenty minutes, third twenty minutes, second hour, and third hour respectively, plotted as in Fig. 2.

—●—●— Whole brain in Ringer solution.
—⊙—⊙— Whole brain in alcoholic Ringer.
—■—■— Grey matter in Ringer solution.
—⊡—⊡— Grey matter in alcoholic Ringer.

Fig. 5, for the second hour of respiration in the Barcroft apparatus, shows the increased oxygen consumption persisting for those animals killed ¼ hour after receiving alcohol, but for all other cases the oxygen usage varies somewhat irregularly on either side of the normal value. On the whole, the tendency is for the whole brain tissue suspended in Ringer to give essentially normal values but for the others to give values rather below the normal except for animals killed 4 hours after receiving alcohol. At that time all the values are approximately normal, as they are in all cases during the third hour of respiration *in vitro* (Fig. 6).

The method of presenting the experimental results which we have adopted

in these figures allows one to compare at once the brains of animals which received alcohol at varying times before death, but which have undergone observation in the Barcroft apparatus for the same length of time, and are, therefore, in approximately the same physiological condition. A consideration of the figures separately, and together, supports the conclusion that the first effect of alcohol is to stimulate the uptake of oxygen by brain tissue, and especially by the grey matter. This stimulation is maximal about 15 minutes after the ingestion of alcohol. This fact emerges whether one is dealing with tissue suspended in Ringer alone or in Ringer *plus* alcohol, but the kind of suspension fluid has evidently an influence on the oxygen uptake both with animals which were killed 2 or 3 hours after receiving alcohol, and with brains which have survived for a similar period in the Barcroft apparatus.

We regard the tissue suspensions in alcoholic Ringer as representing more nearly the conditions subsisting in the living animal. The concentration of alcohol present—about 0·35 % by weight—is approximately that of the blood ½ hour after administration, and with the large dose we gave not much above the blood concentration after 3 hours. Thus the surviving tissue, throughout the period of *in vitro* observation, is kept surrounded by alcohol at very nearly the same concentration as would have obtained in the intact animal. If this view is justified, it appears that the early stimulating effect of alcohol is rapidly followed by a depression.

This depression, however, is most clearly shown in the later charts derived from observations on tissue which has already survived 1 or 2 hours in the Barcroft apparatus. It is necessary, therefore, to examine the figures more closely to ensure that the apparent depression is due to alcohol, and not merely to gradual death of the brain cells during the period of observation.

If the graphs represent what really takes place in the living animal, and are not merely artefacts produced by dealing with dying tissue, then the figures for oxygen uptake should be the same at the same time after alcohol ingestion no matter what proportion of the interval has been spent in the Barcroft apparatus. Thus we have a number of observations on the rate of oxygen uptake 3 hours after alcohol ingestion; in some of these the alcohol was in contact with the brain tissue ½ hour during life and 2½ hours after death of the animal; in others 1 hour during life and 2 hours *post mortem*; and in yet others 2 hours during life and 1 hour *post mortem*. All these observations should give the same value for the oxygen uptake if the tissue is not dying during the period of its incubation in the Barcroft apparatus. This, of course, is rather too much to expect, since even the control tissue shows some falling-off in respiration rate during the second and third hours. We may, however, justifiably expect the values for oxygen uptake at the same time after alcohol ingestion to show variations in the same direction from the corresponding controls, no matter how the time has been made up. Too rigid an adherence even to this rule cannot be enforced in view of the inherent experimental difficulties, but any marked departure from it must be taken as indicating

unreliability of the results, and, therefore, as nullifying any deductions drawn from them.

Analysis of the figures in this way shows in fact that during the first $1\frac{1}{2}$ hours after ingestion of alcohol there is undoubtedly an increased oxygen uptake by whole brain tissue no matter what proportion of that period has been spent in the Barcroft apparatus, and what proportion in the living animal. From $\frac{3}{4}$ to $2\frac{1}{2}$ hours after alcohol ingestion the oxygen uptake varies to some extent above and below the control values, but is substantially normal. During the period $2\frac{1}{2}$ to $3\frac{1}{2}$ hours after alcohol ingestion (beyond which time our results do not justify going) there is general agreement that the rate of oxygen uptake is less than normal.

Similar treatment of the results for grey matter shows that they too are substantially correct.

Discussion.

The interpretation of the experimental results described in the preceding section is a matter of considerable difficulty, and we are not yet in a position to offer any satisfactory explanation of them.

At first sight it seems possible that the alcohol when first presented to the oxidising surfaces of the brain cells is, in effect, an additional metabolite, causing greater percentage saturation of these surfaces and, therefore, greater oxygen uptake. This involves the assumption that the oxidising surfaces are not normally working at full capacity, and this might perhaps be supported, but further explanation along these lines demands assumptions for which no justification at present exists. Thus one might suppose that the alcohol by virtue of its high molecular concentration in the cells becomes more and more preferentially adsorbed (and therefore oxidised), but if this is to explain the later depression in the oxygen uptake one must suppose that, although preferentially adsorbed, the alcohol is more slowly oxidised than the other metabolites, and no really strong evidence exists to suppose this. It is true that, for dogs, Mellanby [1919] has reported a steady oxidation rate for alcohol of 0·185 cc. per kg. body wt. per hour, a rate much less than that at which carbohydrate and fat can be oxidised. This, however, though it prevents *a priori* negativing of the assumption, can hardly be said to support it strongly. Indeed we do not know as yet whether any part of the oxygen uptake in our experiments is really due to alcohol. Information on this point, which should further support or definitely negative the hypothesis we have outlined, is now being sought by determinations not only of the oxygen usage, but also of the respiratory quotient. A difficulty at present known to exist is that, in our experiments, the concentration of alcohol in the brain (and blood) reached its maximum in $\frac{1}{2}$ hour after administration, and though it fluctuated somewhat after that, had fallen only very slightly after a further $2\frac{1}{2}$ or $3\frac{1}{2}$ hours. Unless the adsorption on the enzyme surfaces lagged very considerably behind the appearance of alcohol in the brain, the time sequences of the two phenomena

are difficult to reconcile. On the other hand one must take into account the special nature of the tissue, with its richness in lipoids, a factor which may well affect the rate at which the alcohol is transferred to the intracellular surfaces from the intercellular fluids.

We may tentatively suggest the following hypothesis, chiefly as a basis for further work on the subject.

During the first ½ hour after alcohol ingestion, when the alcohol content of the blood and tissues is increasing, alcohol is being adsorbed on the oxidising surfaces of the brain but chiefly on the hitherto unoccupied areas so that it hardly interferes with the other metabolites. Thus, although alcohol is relatively slowly oxidised, there is a net increase in the rate of oxygen uptake. Now, however, the surfaces approach saturation, and competition begins even for those parts of the surface previously occupied by the usual metabolites. In this competition the alcohol wins by virtue of its maintained high concentration and ready permeability of the cell walls. Hence more and more of the surface becomes covered with alcohol, and less and less with carbohydrate. Consequently, on the assumption of the slow oxidation of alcohol, the rate of oxygen uptake falls. How far it will fall depends on the relative rates of oxidation and on the relative concentration of the different competitors. Such a mechanism, however, can conceivably account for both the early increase and the later decrease in oxygen usage, and can reconcile them with the maintained high concentration of alcohol in the tissues.

It is also necessary to obtain, if possible, some information as to whether alcohol is or is not a normal metabolite. It can be oxidised at any rate by tissues other than brain but there is no proof of which we are aware that the energy liberated by its oxidation can be used for any other purpose than maintenance of temperature. If its energy is utilisable only as heat, then the possibility exists that the apparent stimulation in brain metabolism soon after ingestion of alcohol is really a depression of normal metabolism, the excess oxygen uptake being due to oxidation of alcohol without liberation of energy available for the ordinary brain processes. This hypothesis again meets with difficulties which can only be removed (or shown to be unsurmountable) by further work of the kind we have outlined above, and also with other narcotics.

Finally account must be taken, either alone or in conjunction with some such hypotheses as those just outlined, that some oxidation product of alcohol may be toxic. Such a suggestion, it is true, comes very near to begging the question, unless it can be shown how the toxicity is exerted—as, *e.g.* by the poison being strongly adsorbed but slowly oxidised, or by "poisoning" the enzyme surfaces.

Summary.

Ethyl alcohol at first increases the rate of oxygen uptake of brain tissue. This increase, which is greater in grey matter than in white, lasts for about an hour, and is followed by a fall to slightly below the normal rate. Further work

is needed before these results can be interpreted fully, but it is tentatively suggested that alcohol is adsorbed on the oxidising surfaces, thereby displacing more usual metabolites, but is more slowly oxidised. The initial increase in the rate of oxygen uptake is explained on the assumption that the alcohol first takes up unoccupied parts of the surfaces. The alcohol content of the brain reaches its maximum, under the experimental conditions, in about ½ hour, and has fallen very slightly in 4 hours.

We wish to take this opportunity of thanking Prof. Sydney Smith for suggesting this work, and for his constant interest and helpful criticism during its progress. We also have to thank the Medical Research Council for a part-time grant to one of us (C. P. S.) and the Moray Research Fund of this University and the Henderson Trust for grants which defrayed part of the expenses.

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VIII. CUPROUS GLUTATHIONE.

By NORMAN WINGATE PIRIE.

From the Biochemical Laboratory, Cambridge.

WITH A NOTE ON THE CRYSTALLOGRAPHY OF GLUTATHIONE.

By JOHN DESMOND BERNAL.

(*Received December 22nd, 1931.*)

IN a recent paper Kozlowski [1931] has described the preparation of a compound, containing 16·45 % Cu, by addition of excess of cuprous oxide to a solution of oxidised glutathione in *N*/2 sulphuric acid, filtering and aerating. This compound he assumes to be a derivative of oxidised glutathione, presumably of the composition Cu_2GSSG. In view of this claim it has been thought necessary to reinvestigate this reaction.

Since cuprous oxide is a reducing agent it is to be expected that it will first reduce the oxidised glutathione and then combine with the reduced molecule to form a soluble derivative of the type $(CuSG)_2Cu_2SO_4$ [Pirie, 1931, p. 623], which, on exposure to air, breaks down with the formation of cuprous glutathione and cupric sulphate. There are five independent lines of evidence for this view.

(*a*) A solution of oxidised glutathione will, after the addition of a small amount of cuprous oxide, give a nitroprusside reaction; whereas, after the addition of a cupric salt, it will not give a nitroprusside reaction unless a reducing agent such as KCN is added.

This is not conclusive evidence that the reduction can occur in acid solution; it may only take place after the addition of the ammonia.

(*b*) If a de-aerated solution of oxidised glutathione in *N*/2 sulphuric acid is mixed *in vacuo* with an air-free suspension of cuprous oxide containing not more than two molecules of Cu_2O for each molecule of GSSG a clear blue solution results. This suggests that a cupric salt has been formed. If more cuprous oxide is added the solution turns yellow or green.

(*c*) The specific rotation of a solution of cuprous glutathione prepared by the ordinary Hopkins [1929] method is identical with that of a solution of cuprous glutathione prepared by Kozlowski's method.

(*d*) When titrated with iodine solution in the presence of potassium iodide and acetic acid cuprous glutathione prepared by either method takes up one atom of iodine for each atom of copper, as would be expected if its formula were CuSG. If the formula were Cu_2GSSG there is clearly no reason why, under these conditions, it should take up any iodine at all.

(*e*) A molecule of the type suggested by Kozlowski should, on decomposition with H_2S, give rise either to cupric sulphide or to a mixture of cuprous sulphide and sulphur if at the same time reduced glutathione is formed. I find that reduced glutathione is formed quantitatively while the ratio of copper to sulphur in the precipitate is 2 : 1. The analytical data in support of (*c*), (*d*) and (*e*) are given in the experimental section.

Experimental.

Reduced glutathione was prepared by the method already described [Pirie, 1930], except that for 2 kg. of yeast 120 cc. of ether are now used instead of the 80 cc. which were used in the original method. It is a matter of some importance that there should not be too much peroxide in the ether used since this oxidises the glutathione and renders the precipitation incomplete. In one or two preparations I have replaced the ether by the same amount of phenol with satisfactory results; any substance which will kill yeast cells when present in fairly low concentration and which does not interfere with the subsequent cuprous precipitation would probably do equally well. The killing of the cell appears to be necessary for the liberation of glutathione since I have been unable to find any reagent which will make glutathione diffuse into the fluid part of a suspension of yeast cells without causing a proportionate fall in the viable count of the suspension.

If large crystals of glutathione are not required alcohol may be added to the filtrate from cuprous sulphide as in Hopkins's [1929] method, but crystallisation is improved if the use of alcohol is avoided as in the method which I have already described. By this method crystals 1–2 mm. long are always obtained and they are sometimes as large as $10 \times 2 \times 2$ mm. (See Fig. 1.)

Crystallographic data.

Mr J. D. Bernal has kindly examined these crystals and gives the following description of them, a more complete account will be published shortly in another place.

"The crystals occur in orthorhombic prisms or plates bounded by many narrow faces, chiefly (001) (302), and terminated by (011) (?). The extinctions are straight. No optic picture could be obtained but the γ direction is probably b.

Oscillation and Weissenberg photographs have been taken establishing a cell $a = 28{\cdot}0$, $b = 8{\cdot}74$, $c = 5{\cdot}57$ Å., containing 4 asymmetric molecules with density 1·50 and with the space group $V^4 - 2_12_12_1$. The positions of the molecules in the cell cannot be determined from such a preliminary investigation but from the fact that the ($2n$00) planes are fairly normal it is most probable that they form two layers 14·0 Å. thick and are of a straight chain character."

When preparing cuprous glutathione from pure glutathione it is advisable to heat the solution to about 40° to get a perfectly white product; with crude

preparations or yeast extracts or in the presence of a trace of hydrochloric acid this is unnecessary and no copper or cupric oxide is formed even in the cold. The precipitate is washed on the centrifuge in the usual way and, since a light

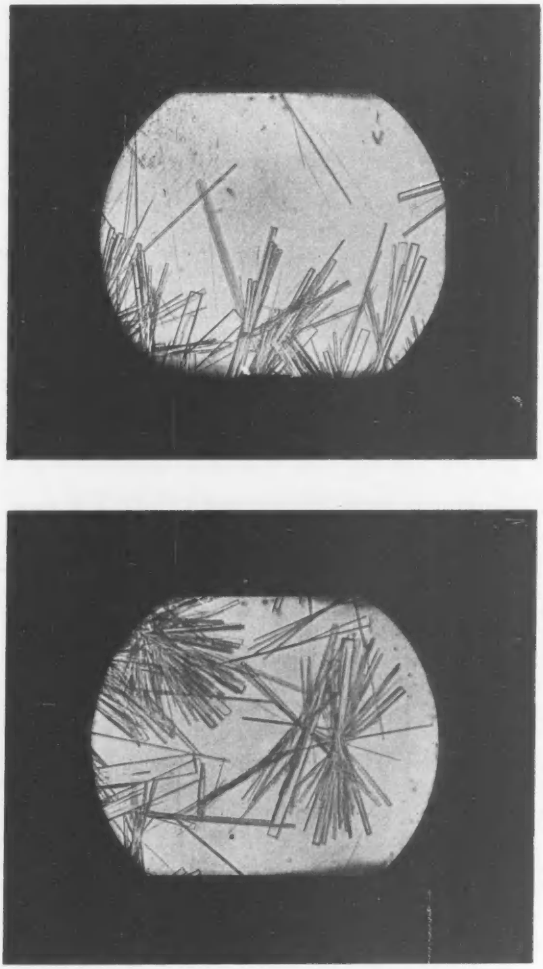

Fig. 1. Glutathione crystals from water. Magnification × 130.

friable product is required, dried in the centrifuge-tube *in vacuo* over phosphorus pentoxide.

Cuprous glutathione (Kozlowski) is prepared from oxidised glutathione by

adding about 4 mols. of cuprous oxide, as an aqueous suspension, to 1 mol. of oxidised glutathione, in the form of a 1 % solution in $N/2$ H_2SO_4. After shaking for about a minute the mixture is filtered by suction and air is then bubbled through the filtrate for an hour or two. This white precipitate is dried like the last. When prepared in this way cuprous glutathione is soluble in an acid cuprous oxide suspension and precipitates again on aeration in the manner already described [Pirie, 1930].

Cuprous glutathione is readily soluble in dilute hydrochloric acid and gives a pale brown solution which is not very stable but apparently gradually dissociates into free glutathione (or its decomposition products) and a copper salt. For example:

89 mg. of CuSG prepared from GSH dissolved in 6 cc. of 1·4 *N* hydrochloric acid.

Time ...	7 mins.	20 mins.	37 mins.	6 hrs.	9 hrs. 40 mins.
Rotation in 2 dm. tube	+1·34°	+1·33°	+1·27°	+0·97°	+0·63°
Time ...	12 hrs. 20 mins.	22 hrs.	31 hrs. 27 mins.	36 hrs.	
Rotation in 2 dm. tube	+0·32°	−0·34°	−1·05°	−1·50°	

The initial reading gives $[\alpha]_{5461}^{16\cdot5^\circ} = +45\cdot1^\circ$.

If stronger acid is used as solvent the rotation, initially low, rises in the course of an hour and then falls again but much more slowly than in the case of the less acid solution. For example:

90 mg. of the same CuSG dissolved in 6 cc. of 4*N* HCl.

Time	5 mins.	9 mins.	28 mins.	50 mins.	85 mins.
Rotation in 2 dm. tube	+0·53°	+0·63°	+0·76°	+0·80°	+0·80°
Time ...	4 hrs. 25 mins.	9 hrs. 28 mins.	12 hrs. 15 mins.	21 hrs. 45 mins.	31 hrs. 20 mins.
Rotation in 2 dm. tube	+0·72°	+0·60°	+0·56°	+0·36°	+0·13°

Reduced glutathione itself when dissolved in 4*N* HCl does not show a comparable change in rotation although the specific rotation in strongly acid solution is − 9° for the mercury-green line instead of − 18·5° as it is in aqueous solution. The rise in the rotation during the first hour is not accompanied by any change in the formaldehyde titration; it has not been investigated further.

When prepared from oxidised glutathione the cuprous compound behaves in an identical manner on keeping in hydrochloric acid solution and has, under the same conditions as before, $[\alpha]_{5461}^{16\cdot5^\circ}$ + 45·4° to + 45·8°.

It is already well known that cuprous mercaptides, suspended in a weakly acid solution, become soluble on the addition of small amounts of a chloride. As might be expected iodides have a similar effect. The cuprous iodide which results is itself held in solution by the thiol compound and does not separate until some of this has been oxidised to the disulphide. On the addition of more thiol compound it again goes into solution but reprecipitates on adding iodine.

58 mg. of cuprous glutathione prepared from GSSG were suspended in 3 cc. of water and 0·5 cc. of glacial acetic acid; addition of 0·2–0·3 g. of potassium

iodide caused complete solution. Using starch as indicator the mixture was titrated with $N/100$ iodine. 15 cc. were required (theoretical 15·7). Cuprous glutathione prepared directly from reduced glutathione showed identical equivalence to iodine.

108·4 mg. of cuprous glutathione prepared from oxidised glutathione were suspended in about 10 cc. of water in a 50 cc. round-bottomed flask. The mixture was decomposed with H_2S, with rigid exclusion of air for 2–3 hours and was then connected to an efficient pump and exhausted cautiously, the flask was kept warm to prevent the solution from freezing. After an hour the cuprous sulphide and any free sulphur that might have formed were removed by centrifuging and the precipitate washed once on the centrifuge. The centrifugates, which were water-clear, were taken nearly to dryness in a vacuum desiccator over sulphuric acid and sodium hydroxide.

The precipitate remaining in the centrifuge-tube contained 5·23 mg. S and 19·4 mg. Cu, giving a Cu : S ratio of 3·78 (calculated for Cu_2S, 3·97). This agreement is as close as could be expected and certainly excludes the possibility that either cupric sulphide or an equimolecular mixture of cuprous sulphide and copper is formed.

The centrifugate which contained glutathione was titrated with $N/1000$ iodine; 5·4 cc., corresponding to 84 mg. of GSH, were required. This indicates that at least 95 % of the glutathione was now in the reduced form.

SUMMARY.

A modification in the method of preparation of glutathione is described.

Crystallographic data and photographs of the crystals are given.

The specific rotation of cuprous glutathione has been measured and evidence is brought forward to show that there is no reason to assume the existence of a cuprous derivative of oxidised glutathione, insoluble in weak acids.

I am deeply indebted to Sir F. G. Hopkins for his interest in this work.

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for The centrifugate, which contained glutathione, was titrated with $N/1000$ iodine; 5·4 cc., etc.

read The centrifugate, which contained glutathione, was made up to 25 cc. and 5 cc. were titrated with $N/100$ iodine; 5·4 cc., etc.

IX. STUDIES IN THE BEHAVIOUR OF THE CARBOHYDRATES AND LACTIC ACID OF THE MUSCLE OF THE HADDOCK (*GADUS AEGLEFINUS*) AFTER DEATH.

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(*Received December 23rd, 1931.*)

It is well known that the changes which occur in fish muscle after death are only moderately retarded by storage at freezing point. They set in before any contamination of the muscle can be demonstrated, an outward manifestation of their occurrence being development of *post mortem* rigor. In investigations on frog muscle, Smith [1929] has shown that lactic acid does not increase above the initial value when the muscle is kept at 0°, but that it does so at − 2·5°, reaching in 48 hours a concentration of 0·62 %. At temperatures lower than this, production of lactic acid in 48 hours diminishes progressively until at − 10° none is formed. The explanation for failure of lactic acid to accumulate at 0° has been found by Smith and Moran [1930] to depend on the fact that the reversible changes which are responsible in living muscle for its disappearance are retained for some time on freezing down to a critical temperature of − 1·6°. On thawing the muscle after it has been exposed to temperatures between this and 0° these reversible changes therefore cause any lactic acid which has accumulated to disappear. Below −1·6° the mechanism of the reversible change is destroyed, so that the lactic acid remains. It seemed of importance to find out whether similar changes in lactic acid concentration occur in fish muscle and if so to determine the critical temperature.

As a first step towards the investigation of this problem it was necessary to obtain further information than at present exists concerning the relationship of lactic acid production to the glycogen content of fish muscle. Although it has been known for some time, through the work of Ritchie [1928], Simpson and Macleod [1927] and Leim, Simpson and Macleod [1927], that lactic acid accumulates and glycogen disappears, the exact relationship between these two processes remains undetermined. To obtain this information was the primary object of the present investigation in which attention has also been paid to other problems, such as the possible nature of the intermediary substances formed between glycogen and lactic acid, and the changes in reaction which occur in fish muscle *post mortem*.

Methods.

Haddock (*Gadus aeglefinus*) weighing about 2 lbs. each were caught by hook and line and as rapidly as possible transferred to tanks of frequently changing sea-water in which they were left for not less than one week before use. After a day or so the captive fish ate mussels readily and were normal in other respects. These precautions were found to be necessary in order to obtain any reasonable constancy in the glycogen content of the fish immediately after being killed.

When fish were required they were quickly netted, killed by stunning, immediately gutted and filleted. Glycogen was determined by Pflüger's method. Lactic acid and free sugar were extracted with 5 % trichloroacetic acid at a temperature of 0°, an aliquot portion of the resulting extract after neutralisation being then precipitated with $\frac{1}{6}$ its volume of 10 % solution of copper sulphate and $\frac{1}{3}$ volume of 10 % suspension of calcium hydroxide. After filtering, or centrifuging, a further aliquot of the filtrate was used for determination of lactic acid by the method of Davenport and Cotonio [1927], and the precipitate was treated by the method recommended by Holmes and Gerard [1929] for determination of free sugar.

In some experiments the free sugar was determined by extracting it repeatedly from the muscle by alcohol—usually 90 %—at 0°. After removal of the alcohol in a warm air stream, the residue was taken up in water, the resulting solution treated with "colloidal iron" and sodium sulphate, filtered and the reducing power measured by the Shaffer-Hartmann [1921] method both before and after hydrolysis with 2·2 % HCl. In a third series of experiments the Holmes and Gerard method [1929] was applied to the "colloidal iron" filtrate. Since much uncertainty exists as to the exact significance of the results obtained by these methods for free sugar it may be of value to give examples comparing those obtained by different methods applied to the same muscle.

Exp. No.	Extractant	%	Further treatment of extract	Reducing substance mg. per 100 cc. Before hydrolysis	After hydrolysis
1	Alcohol	40*	"Colloidal iron"	0·074	0·149
	,,	60	,,	0·072	0·135
	,,	75	,,	0·069	0·105
	,,	90	,,	0·050	0·119
2	,,	40	,,	0·097	0·109
	,,	90	,,	0·049	0·049
3	,,	90	,,	0·050	—
	,,	90	Do. + Holmes and Gerard method	0·0083	—
4	,,	90	"Colloidal iron"	0·066	0·117
	Trichloroacetic acid	5	,,	0·005	—

* In every case 10 g. muscle were finely cut up with sharp scissors in 10 times their weight of the alcohol solutions indicated in the Table. This was conducted in a cold room just under 0° where the suspension was allowed to stand overnight. The extract was either filtered and the residue re-extracted or it was centrifuged and an aliquot portion of the supernatant fluid used.

These results, which are typical, show:

(1) That less reducing substance is extracted as the strength of alcohol increases.

(2) That hydrolysis of the filtrate after precipitation with "colloidal iron" usually causes an increase in reducing power.

(3) That adsorption of the sugar by cupric hydroxide as in Holmes and Gerard's method, gives much lower values than extraction alone.

(4) That the amount of reducing substance directly extracted with trichloroacetic acid is comparable with that obtained when Holmes and Gerard's method is applied to a "colloidal iron" filtrate.

Whether the value of about 0·05 % obtained after treatment with "colloidal iron" or the much lower one of 0·005–0·010 % which is given by the use of Holmes and Gerard's method is to be accepted as representing free sugar is uncertain. On this account both methods have been used in the present investigation.

Results.

Six haddocks were taken from the tank, killed and immediately gutted. They were then packed in broken ice and taken to the laboratory, where glycogen and lactic acid determinations were at once started. The values obtained at approximately 20 minutes *post mortem* are given in Table I.

Table I.

	1	2	3	4	5	6
Glycogen %	0·64	0·51	0·44	0·50	0·53	0·57
Lactic acid %	0·079	0·091	0·082	0·031	0·088	0·037

Since the average percentage of lactic acid is considerably above that recorded for uninjured frog muscle immediately after death, it was thought possible that some *post mortem* change might have already occurred, and attempts were made to prevent this by sudden freezing of the fish. This was done by immersing other fish, immediately after killing, in liquid oxygen contained in an Isovac food carrier. Instead of diminishing, this treatment caused a distinct increase in the percentage of lactic acid, as Table II shows.

Table II.

	1	2	3	4	5
Glycogen %	0·56	0·58	0·61	0·39	0·65
Lactic acid %	0·196	0·116	0·079	0·144	0·140

Twitching of the muscles at the moment of immersion may have been responsible for the increase. The free sugar extracted with 90 % alcohol varied in these fish between 0·034 and 0·055 %. Adding together the values for glycogen, lactic acid and free sugar gives totals varying between 0·67 and 0·84%.

For practical reasons it was of interest to compare the glycogen and lactic acid percentages (Table III) in haddocks obtained from the Fish Market. These particular fish had been caught by means of a trawl net which had been

dragged along the sea bottom for a period of about 3 hours, at any time during which period the fish might have been actually entrapped. The fish had been stowed in ice after catching and had been about 75 hours out of the sea when they were used for the experiment.

Table III.

	1	2	3	4	5	6	7	8	9
Glycogen %	0·007	0·007	0·015	Trace	Trace	Trace	Trace	Trace	Trace
Lactic acid %	0·154	0·142	0·166	0·185	0·140	0·169	0·250	0·106	0·184

Simpson and Macleod [1927] found that the percentage of muscle glycogen in haddocks caught by a trawl line (a line set with about 100 baited hooks and left for between 40 minutes and 1 hour before hauling) was very much less than in those caught meanwhile from the same boat by single line. Often glycogen was absent from the trawl-lined fish, in which also the percentage of lactic acid was much higher than in the hand-lined fish. It was presumed that the struggling of the trawl-lined fish on the hook was responsible for the results then recorded, and it is similarly presumed that the struggling of the fish in their endeavour to free themselves from the accumulating pressure of other fish in the purse of the net is responsible for the absence of glycogen in the results now recorded.

Assuming however that the percentage of glycogen in the trawl-netted fish at the moment of entrapping was similar to that in the tank fish immediately *post mortem* (Table I), much higher values might be expected for lactic acid. Two possibilities may account for the discrepancy, namely, either all the glycogen which disappeared did not reach the lactic acid degree of breakdown, or some of it after being formed into lactic acid was in some way removed.

In view of these preliminary results it was decided to determine the percentages of glycogen and lactic acid in the muscle of fish kept surrounded by ice after removal from the tank. The observations were made at intervals up to 6 days by which time, as has been shown by Dr M. M. Stewart, there is no bacterial contamination of the muscles.

In the earlier experiments 21 haddocks were used and, at the intervals indicated in Table IV, two were removed for determination of glycogen and lactic acid and a third for p_H by the method described by Benson [1928].

Table IV.

Time after death in hrs. Fish kept in ice	Lactic acid %			Glycogen %			p_H
	A	*B*	Mean	*A*	*B*	Mean	Mean
0	—	—	0·068	—	—	0·530*	6·99
12	0·28	0·20	0·240	0·170	0·320	0·245	6·59
24	0·38	0·36	0·370	0·150	0·200	0·175	6·47
35¼	0·13†	0·16†	0·145†	0·027	0·086	0·056	6·65
53	0·33	0·30	0·315	0·000	0·000	0·000	6·69
72	0·16	0·28	0·220	—	—	—	6·65
83	0·19	0·18	0·185	—	—	—	6·67
145	0·10	0·12	0·110	—	—	—	6·56

* Average of fish in Table I.
† No explanation could be found for the exceptionally low value.

The first experiment gave the results shown in Table IV.

The results show that the glycogen disappeared in about 50 hours at which time the percentage of lactic acid had reached a maximum, falling at later periods. The hydrogen ion concentration rose during the first 24 hours after which it fell again slightly and then became constant.

A repetition of this experiment on another batch of fish provided general confirmation of these results, the chief difference being that they still seemed to retain a small quantity of glycogen after 50 hours in ice.

Attention was then focused on the reduction in lactic acid which seemed to occur after about 50 hours in these experiments. Since this could not be attributed to bacterial contamination its occurrence would obviously be of biochemical interest and demand further investigation. But it was necessary to make certain that the reduction was a real one and not accidental, due to variability in the different pairs of fish. It was decided therefore to repeat the observation by using mixed pieces of muscles from several different fish.

As in the previous work, haddocks were taken from the tank and killed, immediately gutted and stored in ice. For each determination, 6 haddocks were taken from the ice and quickly filleted by an expert filleter. The 12 fillets were laid out close together on a board and three cuts were made across them with a sharp filleting knife. Two lots of 12 pieces each were used for lactic acid and glycogen determinations respectively, the samples for analysis being removed in a cold room just under 0° at the intervals indicated in Table V.

Table V.

Time in hrs. Kept in ice	Percentage	
	Glycogen	Lactic acid
0·33	0·420	0·068
3	0·160	0·106
4	0·155	0·099
5	(0·080)	(0·091)
22·75	0·161	0·139
23·75	0·107	0·160
25·25	0·090	0·150
27·25	(0·110)	(0·113)
40	0·046	0·170
48·5	(0·085)	(0·200)
113	0·033	0·166
137	0·039	0·153
140	0·030	0·108
141·5	0·041	0·149

Although the results for both glycogen and lactic acid do not fall in line at successive time intervals, the following general conclusions may be drawn from them.

1. Glycogen disappears very rapidly during the first three hours, after which the decline is a gradual one, small amounts only being left after about the fiftieth hour. It may here be remarked that we are uncertain whether percentages for glycogen lower than about 0·04 are to be reckoned as such.

2. The accumulation of lactic acid during the first three to five hours pro-

ceeds at a rate which is far behind that of the disappearance of glycogen. It then continues more slowly, but fairly steadily (omitting the twenty-seventh hour) until about the forty-eighth hour, after which the evidence suggests a decline.

3. The maximal percentage of lactic acid which appears in the muscle falls far short of the initial percentage of glycogen.

These conclusions confirm those previously drawn by Simpson and Maçleod [1927] from observations on mammalian muscle after being frozen in liquid air, pulverised and then allowed to stand for an hour or so at the temperature of melting ice.

The slow rate of accumulation of lactic acid in comparison with the rapid disappearance of glycogen probably indicates the production of some intermediary substance out of which lactic acid is then very gradually derived. This substance may be of the nature of (1) a lower polysaccharide (dextrin), or a trisaccharide such as has been shown by Barbour [1929] to be the sole product of the hydrolysis of glycogen when pure solutions of this are incubated with a glycerol extract of muscle, or (2) a phosphoric acid ester. Were any of these substances formed they would almost certainly be destroyed during heating with conc. KOH in the Pflüger process for glycogen estimation and it is possible that the production of lactic acid from them is a very slow one. Further work will be necessary to identify the nature of the intermediary substance and in the present investigation we have contented ourselves with ascertaining whether its presence could be revealed by determining the free sugar before and after acid hydrolysis of extracts of the muscle at various periods during the glycogenolysis. As has already been stated (p. 81) the methods available for determination of free sugar in solid tissues do not all yield the same results, which makes their interpretation difficult. In the present case the methods used were either extraction with 40 or 90 % alcohol, followed by treatment with "colloidal iron," or extraction with 5 % trichloroacetic acid.

Table VI.

Mixed samples from live fish.

Extractant %		Sugar g. per 100 g. muscle: Unhydrolysed	Hydrolysed
Alcohol	90	0·066	0·117
Alcohol	40	0·062	0·087
Trichloroacetic acid	5	0·0056	—

Mixed samples from fish kept 20 hrs. in ice.

Extractant %		Unhydrolysed		Hydrolysed	
Alcohol	90	0·054	0·060	0·088	0·076
Alcohol	40	0·062	0·070	0·090	0·110
Trichloroacetic acid	5	0·005	0·005	—	—

Mixed samples from fish kept 24 hrs. in ice.

Extractant %		Unhydrolysed		Hydrolysed	
Alcohol	90	0·062	0·058	0·105	0·104
Alcohol	40	0·054	0·068	0·098	0·136
Trichloroacetic acid	5	0·003	0·003	—	—

The results given in Table VI were those most recently obtained, using mixed samples of muscle from several haddocks. They show that there is no difference in the amounts of sugar before and after hydrolysis in fresh muscle and in muscle which has stood for 20 to 24 hours surrounded by ice.

The increase in reducing power after acid hydrolysis may depend on the presence of dextrins, trisaccharide or phosphoric acid esters, but in any case it seems clear that none of these can have accumulated in the muscle as a result of the rapid initial breakdown of glycogen.

Conclusions.

1. When adequate precautions are taken against struggling of the fish during catching the muscles of the haddock (*Gadus aeglefinus*) immediately after removal usually contain from 0·4 to 0·6 % glycogen.

2. As estimated by its reducing power, the free sugar averages 0·05 % when extracted by 40–90 % of alcohol but is only about 0·01 % when adsorbed from the aqueous solution of an evaporated alcoholic extract by copper sulphate and lime or extracted by trichloroacetic acid. The reducing power becomes approximately doubled by acid hydrolysis of the alcoholic extracts.

3. The immediate lactic acid averages 0·08 % but individual variations are considerable.

4. When the fish after killing is kept in broken ice the following changes occur.

(*a*) Glycogen disappears very rapidly during the first 3 hours and then more slowly, attaining a value of less than one-tenth of its original in from 20 to 24 hours.

(*b*) Lactic acid steadily accumulates, attaining a maximum value of from 0·160 to 0·170 % in 36 to 40 hours, and thereafter remains steady or possibly undergoes slight reduction.

(*c*) No significant changes occur in the free sugar or that produced by hydrolysis.

(*d*) The p_H gradually changes from approximately 7 to 6·45, which it reaches when the lactic acid is at the maximum. It subsequently rises to about 6·65 and remains steady.

5. Since the glycogen disappears much more rapidly than lactic acid accumulates, and since the concentration of the latter at its maximum does not correspond to more than two-thirds of the glycogen originally present, some intermediary substance must accumulate in the muscle. This does not appear to be of the nature of a dextrin or a trisaccharide since no increase was observed in reducing sugar either before or after hydrolysis of extracts made with various percentages of alcohol.

The author wishes to thank Prof. J. J. R. Macleod for his help and interest throughout the investigation.

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X. NOTE ON THE USE OF SINALBIN AS AN INDICATOR.

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(*Received December 23rd, 1931.*)

Sinalbin, the glucoside of White Mustard (*Sinapis alba*, Boiss.), develops an intense yellow colour with alkalis, which disappears on adding an excess of acid [Will, 1872]. This property renders it a useful indicator. It is prepared [Will, 1872] by extracting 400 g. of the finely ground seeds in a large Soxhlet apparatus with carbon disulphide until the extract is free from the yellow essential oil. The residue (200 g.) is dried in air and boiled for 30 minutes with 500 cc. of 95 % alcohol to inactivate enzymes; the alcoholic liquid is filtered hot, and, on cooling (preferably in ice-water), the glucoside crystallises out. A further yield may be obtained by a second extraction with hot alcohol. The crystals are filtered off, washed with a little ether, and twice recrystallised from hot 98 % alcohol; yield 4–5 g. The stock indicator solution is of 0·5 % strength; solutions in dilute (30 %) alcohol keep indefinitely, while aqueous solutions are liable to attack by moulds. About 1–2 cc. of this solution are added to every 100 cc. of liquid under examination.

Properties.

p_H range: 6·2 (colourless)–8·4 (yellow)
Indicator exponent (p_K): 8·2 at 18°

Salt error:	KCl normality	< 0·38	0·38	0·46	0·54
	Correction	Nil	+0·02	+0·06	+0·15
Protein error:	Protein	2 % caseinogen (Hammarsten)	2 % natural egg-white		4 % ox-blood serum
	Correction	− 0·09	+0·03		+0·04

The p_H values were determined electrometrically with the quinhydrone electrode and Cole's Direct Reading Potentiometer (Cambridge Instrument Co.): Sørensen's phosphate buffers were used throughout.

Sensitivity.

Sinalbin gives a sharp end-point with strong acids and bases down to dilutions of $N/100$; with weak acids and bases it may be used down to $N/10$ concentration. For comparison with phenolphthalein, the factor of an approximately $N/10$ solution of barium hydroxide was determined volumetrically

against weighed amounts of pure acid, and the figure checked by precipitating the barium as sulphate. Titrations in each case were carried to the first appearance of the indicator colour.

Standardisation of $Ba(OH)_2$.

		Mean normality
Gravimetric		0·860
Volumetric		
(*a*) With sinalbin:	Against salicylic acid	0·862
	Against succinic acid	0·862
(*b*) With phenolphthalein:	Against salicylic acid	0·856
	Against succinic acid	0·858

Sinalbin is affected by carbon dioxide in the cold, but may be used for the estimation of carbonates in boiling solution: since, however, it is decomposed by hot alkalis the carbonate must be run into a known excess of hot acid, and not *vice versa*. Its sensitivity and p_H range should make it useful for the microtitration of some alkaloids, particularly quinine. It is suitable for use with ammonia.

SUMMARY.

Sinalbin, the glucoside of White Mustard (*Sinapis alba*), may be used as an indicator. The p_H range is 6·2 (colourless)–8·4 (yellow): the salt and protein errors are small. It is suitable for the titration of weak acids and bases, and may be used with ammonia.

I wish to express my thanks to Mr S. W. Cole for his advice and encouragement.

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XI. METABOLISM OF NORMAL AND TUMOUR TISSUE.

V. A NOTE ON THE CARBON DIOXIDE RETENTION OF SERUM: ITS USE IN THE MEASUREMENT OF TISSUE GLYCOLYSIS.

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(*Received December 24th, 1931.*)

In manometric measurements of tissue metabolism in serum or similar fluids the problem of allowing for the CO_2-combining properties of the medium is constantly arising. On the one hand the increase of CO_2 tension due to respiration leads to a portion of the extra CO_2 being bound by salts of weak acids (*BP*), including particularly the alkali proteinates, of the serum according to the equation:

$$BP + H_2CO_3 = BHCO_3 + HP \qquad \text{.....(1).}$$

On the other hand, if as a result of metabolic processes a strong acid (*e.g.* lactic acid, *HL*) is added to the medium, the CO_2 liberated from bicarbonate is less than that equivalent to the amount of acid added, since a portion of the lactic acid reacts with the salts of weak acids as follows:

$$BP + HL = BL + HP \qquad \text{.....(2).}$$

These considerations have been made the basis of a theoretical treatment of the problem by Warburg [1925], in which for simplification he uses a "serum model" consisting of a solution of a single weak ampholyte (glycylglycine, acid dissociation constant, $K = 4 \times 10^{-8}$), in Ringer solution of the bicarbonate concentration of serum. Such a "model" was found by Warburg to bind CO_2 chemically to about the same extent as serum if the total concentration of dipeptide were made about equal to that of carbonic acid. Using this model, Warburg has developed two sets of equations representing the amount of CO_2 retained by this system for a given change in the pressure of free CO_2. The former of these refers to the case when the acid added to the serum is carbonic acid, the latter to that when the acid is lactic or other strong acid.

Since in practice it is unusual to find either of these conditions divorced from the other, a compromise is necessary and one or other of the two sets of equations must be adopted as an approximation, according as the production

of carbonic acid (most normal tissues) or of lactic acid (tumours and certain pathological tissues) predominates.

In Part III of this series a method was described for the manometric measurement of respiratory CO_2, respiration, R.Q. and aerobic glycolysis of tissues in bicarbonate-media, including serum. The method there described for measurement of respiratory CO_2 was completely independent of any of the above considerations. That described for measurement of glycolysis in serum, however, was based on the Warburg [1925] principle. For each measurement of tissue glycolysis this method involves a separate control experiment in which the serum must be acidified with an amount of acid nearly equivalent to that produced in the main experiment. At the same time in another vessel an exactly equal amount of acid is added to a simple bicarbonate-Ringer solution. In the latter case the amount of CO_2 liberated is, of course, equivalent to the acid added, and is thus greater than with serum, the difference being a measure of the amount of the retained CO_2. This measurement of retention, however, as Warburg particularly emphasises, is valid only for the exact conditions of the acidification experiment; even the vessel used must be the same one as in the main experiment.

In practice it is convenient to employ the same specimen of serum for a number of measurements, and much time would be saved by a method involving no such separate determination of retention following every main experiment. For this purpose some linear function of the concentration of undissociated protein HP, the change of which (ΔHP or $-\Delta BP$), according to equations (1) and (2), is the measure of the retention, would possess the advantage of making possible a direct interpolation over the whole physiological range.

Such a relationship exists between HP and the p_H of normal serum. Van Slyke, Wu and McLean [1923], found that the empirical equation:

$$mM.\,(BP) = 0{\cdot}068\,P\,(p_H - 4{\cdot}80) \qquad \ldots\ldots(3),$$

holds for dialysed horse serum, where P ($= HP + BP$) is the total concentration of protein (g. per litre serum).

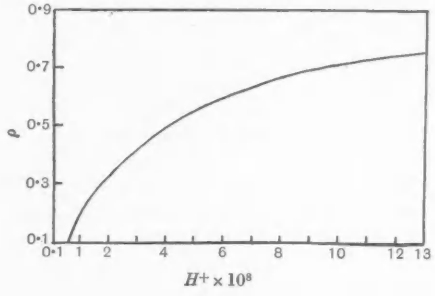

Fig. 1.

A somewhat similar result follows from a consideration of Warburg's serum "model," containing a total concentration of dipeptide c, of acid dissociation constant K and at a hydrogen ion concentration H^+. If in such a model H^+ changes to $H^+ + \Delta H^+$, the corresponding retention (*i.e.* increase in concentration of undissociated dipeptide, HP) is given [Warburg, 1925] by $\Delta HP = \Delta\rho . c$; where $\rho = \frac{H^+}{H^+ + K}$ = Michaelis dissociation residue of the dipeptide.

Such a relationship, plotted graphically, gives a curve of the shape shown in Fig. 1.

Rewriting the above equations we have:

$$H^+ = K . \frac{\rho}{1-\rho},$$

whence

$$\frac{d\rho}{dH^+} = \frac{(1-\rho)^2}{K},$$

and therefore

$$\frac{d\rho}{d\,(p_H)} = -\frac{d\rho}{dH^+} \times \frac{dH^+}{\log_{10} H^+} = -\log_e 10 . \frac{d\rho}{dH^+} \cdot \frac{dH^+}{d\,(\log_e H^+)}$$

$$= -2{\cdot}3 \frac{d\rho}{dH} . H^+$$

$$= -2{\cdot}3 \frac{(1-\rho)^2}{K} . K \frac{\rho}{1-\rho}$$

$$= -2{\cdot}3\,(1-\rho)\,\rho \qquad \text{.....(4).}$$

Table I and Fig. 2 show that in the case of Warburg's "serum model" the

Table I.

ρ	p_H	Δp_H	$\frac{\Delta\rho}{\Delta p_H}$	$\frac{\Delta\rho}{\Delta H^+}$
0·40	7·574			
		−0·0890	−0·56	0·082
0·45	7·485			
		−0·0872	−0·57	0·069
0·50	7·398			
		−0·0871	−0·57	0·057
0·55	7·311			
		−0·0889	−0·56	0·045
0·60	7·222			

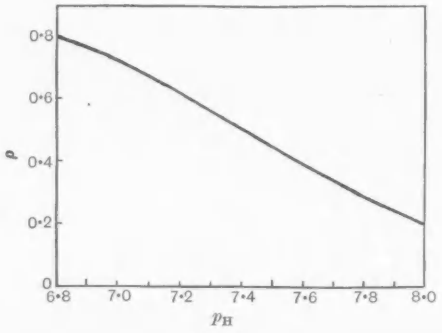

Fig. 2.

value of $\Delta\rho/\Delta p_H$ reaches a maximum value at the point of inflexion of the ρ–p_H curve, but is nearly constant over a considerable range on either side of this point, the retention-p_H curve being nearly linear between about p_H 7·7 and 7·1.

The practical convenience of this logarithmic method of representation is clear. It is only necessary to determine the value of retention ($= \Delta\rho.c$ in the case of the dipeptide model) corresponding to a suitable finite change of p_H in order to be able to interpolate with good accuracy for other values of Δp_H, a separate determination of retention for the conditions of each individual experiment being therefore unnecessary.

When the retention curve of serum is plotted in the same way as in the above example with the "serum model," the nature of the conditions necessary for the similar behaviour of the two systems in retaining CO_2 over the same range of p_H is clearly shown. The conditions required for the dipeptide in the "serum model" are that its point of half-neutralisation should lie at about the mid-point of the range required (*i.e.* its p_K should be nearly equal to the value of p_H at this point), and that the total concentration, c, of dipeptide should be so chosen that in this range the slope of its retention-p_H curve $\left(= c.\frac{\Delta\rho}{\Delta p_H}\right)$ is approximately equal to that of the HP-p_H curve for serum.

The linear nature of the retention-p_H curve for serum rests on experimental evidence. The measurements of Van Slyke, Wu and McLean [1923] were made upon serum which had been dialysed until free from salts, and to which known amounts of Na_2CO_3 had then been added, so that the concentration of total base was thus defined. The empirical equation (3) was found to hold between BP and p_H: the measurements applied to two different specimens of horse-serum and covered a range of p_H 7·07 to 7·74.

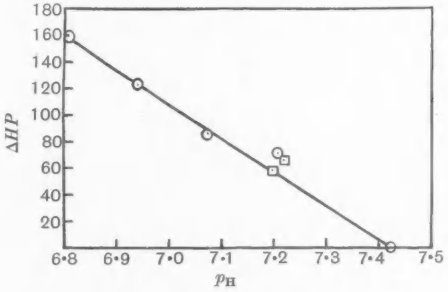

Fig. 3. Normal horse-serum.

In order to establish the applicability of this result for normal serum, we have carried out similar experiments on undialysed serum. In this case the total amount of base present was unknown, hence in the curves ΔHP is plotted against the p_H; the results are shown graphically in Fig. 3. The measurements were made at 38° in vessels attached to Warburg manometers, the manometric

fluid being Clerici solution [Dickens and Šimer, 1931]. The serum (1·5 cc.) was previously equilibrated with gas-mixtures containing known percentages of CO_2. By adding an excess of strong acid, the bicarbonate content was determined manometrically. In some vessels a weighed volume of suitably diluted tartaric acid, which had previously been evaporated in a side-bulb attached to the conical vessel containing the serum, was used for acidification [Warburg, 1925]. In this case the strength of the acid used was controlled by addition of the same volume of tartaric acid to $NaHCO_3$-salt solution. From the resulting pressure readings the CO_2 liberated was calculated. The difference of these amounts gives the amount of carbonic acid combining according to equation (1) above, and hence is equal to the retention, ΔHP. The initial and final p_H of the serum were determined by (*a*) CO_2 analysis by Krebs's method of the original gas-mixtures used and (*b*) the bicarbonate determinations. p_K was taken as 6·10, and the p_H calculated by the Henderson-Hasselbalch equation. The curve (Fig. 3) shows that the linear relationship of HP and p_H holds for undialysed serum also, at least within the limits of accuracy of these experiments, which are similar to those prevailing in the actual manometric measurement of tissue metabolism [Dickens and Šimer, 1931].

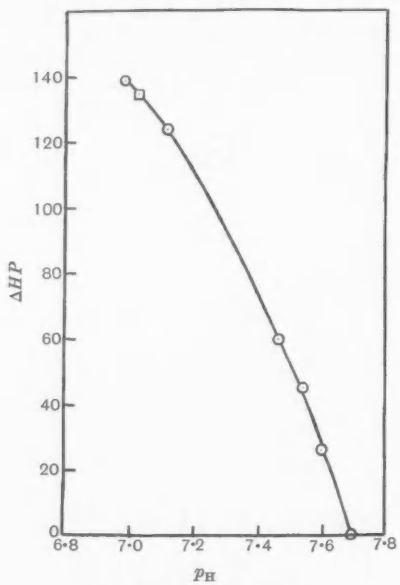

Fig. 4. Dialysed horse-serum.

The measurements on normal serum are somewhat less accurate than those on dialysed serum (Fig. 4), at least for small values of ΔHP, since the accurate

measurement of a relatively small change in a large total amount of combined CO_2 is a matter of difficulty.

In the case of dialysed serum this difficulty can be artificially overcome by adding an abnormally low amount of base to the dialysed serum before equilibrating with the gas mixture. Under the conditions of our experiments, for example, this permits of the use of Brodie fluid (sp.gr. 1·033) as the manometric fluid instead of Clerici solution (sp.gr. approx. 4), with a corresponding fourfold increase in sensitivity. Fig. 4 shows that in the case of dialysed serum over the range of p_H studied the relationship between p_H and CO_2 retention is of similar type to that calculated for the "serum model" and also resembles that found for normal serum.

Practical application to measurement of glycolysis or acid consumption by tissues.

Determination of the retention-p_H curve.

Two methods are practicable: (1) the serum is saturated with gas mixtures containing different known percentages of CO_2, and the bicarbonate contents are determined by acidification with excess of strong acid (points thus determined are shown as squares in Figs. 3 and 4); or (2) the serum is acidified slightly with a known amount of strong acid and the CO_2 evolution is measured.

Of these methods the second is usually more convenient, since a liberal supply of gas mixture is necessary for saturation, and in this method only one sample of gas is required.

The same vessels are used as in the measurement of respiratory quotient in serum [Fig. 1, Dickens and Šimer, 1931]. The manometric fluid is Clerici solution. The vessels are first weighed clean and dry, without stoppers, and into the side-bulbs, *B*, suitable amounts of a dilute tartaric acid solution are pipetted and the amounts checked by weighing to the nearest milligram. The protocol at the end of this paper indicates some suitable amounts of acid. The solution is then evaporated by placing the vessels in the oven at 100°, and in the meantime the serum is approximately saturated with the gas mixture (oxygen with about 5 % vol. CO_2) from a cylinder of the compressed gas. 1·5 cc. of the serum is then carefully pipetted into each vessel (inner part *A*) and in order to control possible slight changes of vapour pressure on acidification a measured volume of saline may be measured into the outer compartment, *C*. Two further vessels are required, of which one is used to control the strength of the tartaric acid used, and contains instead of serum 1·5 cc. of a simple $NaHCO_3$-salt solution: the other vessel contains an excess of 50 % solution of tartaric acid in bulb *B*, and is used for determination of the intial bicarbonate concentration of the serum. All the vessels are connected to the gas supply and shaken in the bath at 38° in a stream of the moist gas for 20 minutes, as was described in detail in Part III. The gas stream is then stopped, the levels in the two limbs of the manometer are made equal and, after the

equilibration has been verified by the constancy of the manometer readings, the acid is washed thoroughly into the main contents of the vessels by removing them from the bath and tipping backwards and forwards.

An example showing the order of magnitude of the resulting pressures and the mode of calculation is given below.

It should be emphasised that the approximately linear shape of the curve should not be assumed except when normal serum is used as the medium. In any case, however, the construction of such a curve merely involves the determination of a few pairs of values of ΔHP and p_H, and is not more troublesome than the determination of retention by direct comparison, since once the curve has been constructed the applicability of the present method is much wider. Serious error may be introduced if the medium used is not kept free from bacteria and, if a large batch of serum is to be used for a series of experiments, it should be passed through a Berkefeld filter and stored in small sterile containers in a refrigerator until required for use.

Application to the measurement of tissue glycolysis.

By the method of measurement of tissue metabolism which has already been fully described [Dickens and Šimer, 1931] the following quantities are directly measured. (For convenience in calculation all pressures are measured in mm. Clerici solution of which $\frac{(760 \times 13{\cdot}60)\text{ mm.}}{s} = P_0 = 1$ atmosphere, where s is the sp.gr. of the Clerici solution. Concentrations are measured throughout in mm.^{3} per 1·5 cc. serum.)

Initial partial pressure of total CO_2 (at t_0)	$=H_0$		
Final " " "	(at t_1)	$=H_1$	
Initial total $BHCO_3$		$=B_0$	$=k_0 . H_{B_0}$
Final "		$=B_1$	$=k_1 . H_{B_1}$

where k is the respective vessel constant for CO_2, and H_{B_0}, H_{B_1} are the actual pressure changes on liberation of bound CO_2 by acidification at times t_0 and t_1.

From these figures the initial and final p_H of the solution may be at once calculated. For this purpose we assume $p_K^{38°}$ for serum = 6·10 [Hastings, Sendroy and Van Slyke, 1928] and $\alpha_{CO_2}^{38°} = 0{\cdot}51$ [Van Slyke, Sendroy, Hastings and Neill, 1928].

The calculation of the amount of dissolved CO_2 follows simply from the relationship $H = H_{gas} + H_{CO_2} + H_B$. Since the partial pressure of free CO_2 ($H_{gas} + H_{CO_2}$) is equal to $H - H_B$, we may write at t_0 dissolved $CO_2 = \frac{H_0 - H_{B_0}}{P_0} . 1500 . 0{\cdot}51$ (mm.^{3} per 1·5 cc. serum), and at t_1 dissolved $CO_2 = \frac{H_1 - H_{B_1}}{P_0} . 1500 . 0{\cdot}51$.

Hence, initial p_H at t_0 (vessel 0) $= p_{H_0} = 6{\cdot}10 + \log \frac{B_0}{\frac{H_0 - H_{B_0}}{P_0} . 1500 . 0{\cdot}51}$,

and final p_H at t_1 (vessel 1) $= p_{H_1} = 6{\cdot}10 + \log \frac{B_1}{\frac{H_1 - H_{B_1}}{P_0} . 1500 . 0{\cdot}51}$,

$$- \Delta p_H = p_{H_0} - p_{H_1} = \log \frac{B_0}{B_1} + \log \frac{H_1 - H_{B_1}}{H_0 - H_{B_0}} \qquad \text{.....(5).}$$

The value of Δp_H being thus known for every experiment, the corresponding retention, ΔHP, is read off at once from the correct region of the retention curve for the specimen of serum used. Here ΔHP is the change between t_0 and t_1 in the amount of base bound by buffers other than bicarbonate contained in the serum. In the absence of buffers other than bicarbonate in the medium, the amount of fixed acid, x_G, formed by the tissue would be exactly equivalent to the change in bicarbonate content x_b, according to the equation

$$HL + BHCO_3 = BL + H_2CO_3 \qquad \text{.....(6).}$$

In serum, however, the reactions shown in equations (1) and (2) occur. An amount of base equivalent to ΔHP is thus yielded, by which amount the fall in the bicarbonate content due to (6) is diminished. In this case, therefore, $x_b = x_G - \Delta HP$. The value of aerobic glycolysis, $Q_{CO_2}^{O_2}$ (mm.^{3} per mg. dry weight of tissue per hour), is then given directly by

$$Q_{CO_2}^{O_2} = \frac{x_G}{m \,.\, t} = \frac{x_b + \Delta HP}{m \,.\, t},$$

where m is the dry weight of the tissue in mg. and t the duration of the experiment $(t_1 - t_0)$ in hours.

It should be noted that before the values of H_0 and H_1 are inserted in the above equations they should be corrected for the slight pressure change (about -2 mm. of Clerici solution) found necessary in the use of Krebs's method for CO_2 analysis [Dickens and Šimer, 1931].

Summary.

(1) The retention of CO_2 by serum shows an approximately linear relationship to the change of p_H over the range studied.

(2) This result applies to both normal and dialysed serum, and also to the "serum model" of Warburg.

(3) The application of the above observations to the measurement of production or consumption of organic acids by tissues in serum is described.

One of us (F. D.) is a member of the Scientific Staff of the Medical Research Council, and the other (F. Š.) wishes to make acknowledgments to the Halley-Stewart Trust Fund for a scholarship.

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Van Slyke, Sendroy, Hastings and Neill (1928). *J. Biol. Chem.* **78**, 765.
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PROTOCOLS.

(1) *Determination of retention-curve for serum.*

Horse-serum (inactivated) equilibrated with oxygen containing 4·5 % CO_2 ($H^{corr.}_{gas}$ = 110 mm. Clerici solution of sp.gr. 3·976 : P_0 = 2600; $P_0 - P_W$ = 2430 mm.). Temperature 38°. See Fig. 3.

Vessel ...	11	7	9	10	11	11
Experiment	Bicarbonate determination	Acidification experiment				Control on tartaric acid solution
Volume of serum (cc.)	1·5	1·5	1·5	1·5	1·5	1·5 cc. $NaHCO_3$-Ringer solution
Contents of bulb	0·1 cc. 6 *N* lactic acid	0·203	0·303	0·406	0·494	0·299
		g. tartaric acid solution (approx. *N*/20): evaporated to dryness before experiment				
Vessel constant for CO_2 (k_{CO_2})	6·29	6·14	6·70	6·56	6·29	6·29
Pressure on acidification (*h* mm.)	H_{B_0} + 108·5	*h* + 27	+ 40	+ 53·5	+ 66·5	+ 55·5
ΔCO_2 ($= hk_{CO_2}$ mm.³)	—	166	268	351	418	349
CO_2 equivalent to tartaric acid (mm.³)	—	237	354	474	577	—
ΔHP (mm.³)	—	**71**	**86**	**123**	**159**	—
$BHCO_3$ (mm.³/1·5 cc.)	B_0 683	*B* 517	415	332	265	—
p_{CO_2} (mm.)	110	137	150	163·5	176·5	—
$H_2CO_3 = \frac{p}{P_0}.0{\cdot}51.1500$ (mm.³/1·5 cc.)	32·4	40·3	44·1	48·2	51·9	—
$p_H = 6{\cdot}10 + \log \frac{(BHCO_3)}{(H_2CO_3)}$	7·424	7·208	7·073	6·938	6·808	—

(2) *Calculation of aerobic glycolysis of liver-tissue in serum.*

The following calculation is for the protocol at the end of Part III [Dickens and Šimer, 1931]. Time of experiment, 3 hours. The serum, gas-mixture and Clerici solution were the same as in (1) above.

Vessel	7	9	10	11
Vessel constant (k_{CO_2})	6·17	6·72	6·60	6·31
Dry weight of tissue (mg.)	15	18·7	21·2	21·3
$\Delta BHCO_3$ during experiment (x_b)	—	+ 3	+ 3	—
$H_0^{corr.}$ (mm.)	233	—	—	232·5
H_{B_0}	113	—	—	111
$H_1^{corr.}$	—	278	284·5	—
H_{B_1}	—	103·5	105·5	—
$H - H_B$	120	174·5	179	121·5
$\Delta p_H = -\log \frac{B_0}{B_1} - \log \frac{H_1 - H_{B_1}}{H_0 - H_{B_0}}$		− 0·163		− 0·171
ΔHP from curve (Fig. 3) mm.³		+ 42		+ 44
$x_G = x_b + \Delta HP$		+ 45		+ 47
Aerobic glycolysis, $Q^{O_2}_{CO_2}$ (mm.³ per mg. dry weight per hr.)		+ 0·80		+ 0·74

XII. THE NATURE OF PLASTEIN.

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(*Received December 25th, 1931.*)

PLASTEIN, which is precipitated from concentrated solutions of the products of peptic digestion of proteins by the action of pepsin at p_H 4, was discovered by Danilewski [1886] who considered it to be a product of synthesis. Numerous workers have since given the subject their attention, the most noteworthy being Wasteneys and Borsook [1924, 2]. These authors [1930] consider plastein to be a protein produced by the synthetic activity of a proteolytic enzyme.

The evidence in favour of the view that plastein is a synthetic product has been admirably brought together by Wasteneys and Borsook [1930] and need not be repeated here.

The weight that should be attached to this evidence may, however, be considered briefly.

The work of Okunew [quoted by Sawjalow, 1901] indicates anhydride formation, but in no way proves the formation of complex molecules from simpler ones, while that of Sawjalow [1908] concerning the order of the "Danilewski Reaction" is invalidated, as pointed out by Wasteneys and Borsook [1930], since the conditions were suboptimal. His titration experiments are valueless on account of many doubtful assumptions made as to the basicity of plastein and the fact that no steps appear to have been taken to mask the effect of the free amino-groups.

Comparisons drawn between plastein and native proteins in respect of such properties as solubility relations, precipitation reactions, colour reactions and digestibility by enzymes are suggestive rather than conclusive, and the same may be said of the biological evidence [*cf.* Beard, 1927; Hermann and Chain, 1912; von Knaffl-Lenz and Pick, 1913].

The decrease in free amino-groups, while undoubtedly well substantiated, is susceptible of other interpretations than that of proof of synthesis. Further splitting of proteoses or peptones present in the substrate, accompanied by cyclic anhydride formation, might well lead to such a decrease, a product being formed which by reason of its structure is almost insoluble at neutrality. Such a substance would probably contain a smaller percentage of its nitrogen as amino-nitrogen than do proteoses or peptones.

Wasteneys and Borsook submitted samples of a digest in which different amounts of plastein had been formed to a process of fractional analysis devised by them [1924, 1]. The composition of the original digest was determined

by the author by extrapolation of curves obtained by plotting the experimental results of Wasteneys and Borsook against their experimentally determined percentages of plastein-N formed. The theoretical percentages of proteose-N, peptone-N and subpeptone-N corresponding to each experimentally determined percentage of plastein-N could then be calculated on the assumption that the statements of Wasteneys and Borsook were true. The values so calculated were plotted against percentages of plastein-N. In Figs. 1 and 2 the unbroken lines represent the theoretical values while the broken curves show the experimental results of Wasteneys and Borsook.

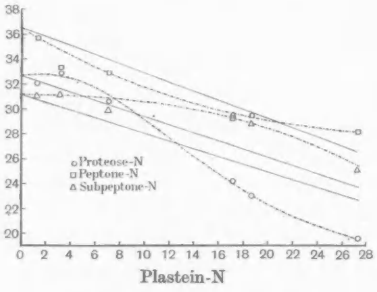

Fig. 1. Series I [Wasteneys and Borsook, 1924, 2].

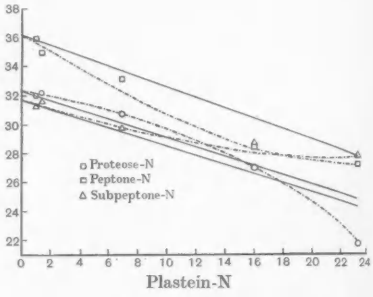

Fig. 2. Series II [Wasteneys and Borsook, 1924, 2].

The wide divergence of the broken curves in Figs. 1 and 2 from the corresponding unbroken ones shows that the assumption made by Wasteneys and Borsook as to the value of their results as evidence for synthesis is not justifiable. All that can be concluded is that, as plastein formation proceeds, all three fractions progressively decrease in concentration, but the question as to whether any one of them enters into the composition of plastein or is decreased as a result of further splitting must remain unanswered.

The conclusion which emerges from a critical examination of the evidence put forward to show that plastein is a synthetic substance is that no unequivocal evidence on the matter has been obtained. The view which regards plastein as a protein is open to doubt.

Present investigation.

In the investigation about to be described the following two questions were kept in view.

1. Is plastein a synthetic product produced by enzyme action?
2. If not, what is plastein and how is it formed?

A definite answer to the first question is afforded if the molecular weight of plastein be known, and hence steps were taken to obtain this information.

The work undertaken in an attempt to answer the second question falls

under two heads. Firstly, a determination of the nitrogen distribution of plastein was carried out in order that the analysis might be compared with that of the protein from the digestion products of which the plastein was prepared. Secondly, an attempt was made to fractionate the phenyl *iso*cyanate derivative of plastein in the hope that, should plastein be found to consist of a mixture of relatively simple substances, some information as to their nature could be obtained.

Preparation of plastein.

The protein from which the peptic digests necessary for plastein formation were prepared was B.D.H. egg-albumin powder. Merck's pepsin powder was added in 0·125 % concentration to 2·5 % albumin solutions in dilute hydrochloric acid at p_H 1·7. When digestion at 37° had proceeded to the point at which no material precipitable by trichloroacetic remained, the enzyme was inactivated by heating at 100° for some time and the digest filtered. The liquid was concentrated under reduced pressure at 50° to about one-twentieth of its original volume. The product obtained after filtering once more was a reddish-brown syrupy liquid, the p_H of which was adjusted to 4 by addition of sodium hydroxide.

In order to prepare specimens of plastein Merck's pepsin powder was added to 2 % concentration and the mixture incubated in presence of toluene for one week. The heavy suspension of plastein was then filtered off, and washed on the filter with hot chloroform water until the washings ran clear and colourless and gave no test for chlorine ions. After drying *in vacuo* over calcium chloride for two weeks, the product being ground in a mortar at intervals, a friable yellowish-white powder was obtained. Specimens prepared in this manner were designated by *A*.

Later, it was found that slightly better yields were obtained if the suspension of plastein first obtained were poured into a large volume of boiling distilled water, the resulting suspension being allowed to settle, the clear top-liquor syphoned off, the residue filtered and the precipitate treated as in the former method. Such specimens were designated by *B*.

One specimen of *A* was suspended successively in absolute alcohol and absolute ether and, after separating by centrifuging, was finally dried *in vacuo* for one week. This specimen was submitted to ultracentrifugal analysis.

The molecular weight of plastein.

Consideration of the problem of the determination of the molecular weight of plastein made it evident that the only practicable method of carrying out such a determination was by use of the ultracentrifugal method developed by Svedberg and applied by him during the last few years to the investigation of the molecular weights of the proteins [*cf.* Svedberg, 1930].

Accordingly a specimen of plastein prepared from a peptic digest of egg-albumin in the manner described above was submitted to Prof. Svedberg who

very kindly consented to carry out the determination. Prof. Svedberg's report is given verbatim:

"We have now run your plastein in the ultracentrifuge. It was dissolved in phosphate buffer of p_H 9·2 (0·032 *M* in Na_2HPO_4 and 0·001 *M* in NaOH) and centrifuged for 2½ hours at a temperature of 21·1° and at a speed of 42,500 r.p.m. corresponding to a centrifugal force about 100,000 times gravity. No sedimentation took place, indicating that the molecular weight of plastein is low. As an upper limit 1000 can be given. Probably the molecular weight is only a few hundred."

The possibility that under these conditions plastein should undergo alkaline hydrolysis, resulting in the breakdown of originally large molecules into molecules which are not sedimented in the ultracentrifuge, is remote. This determination appears to afford conclusive evidence that plastein is not a protein but rather a substance of comparatively low molecular weight.

The nitrogen distribution of plastein.

The procedure used by Plimmer and Rosedale [1925] in a study of the nitrogen distribution of the proteins of hens' eggs was adopted in the present instance, since their modifications of the original Van Slyke technique considerably shorten the process. Their results indicate that under carefully controlled conditions concordant results can be obtained.

In Table I the mean results for plastein are expressed as percentages of total nitrogen, the results of Plimmer and Rosedale for egg-white being given for comparison. Cystine estimations were not made.

Table I.

		Plastein Mean g. N/100 cc.	Plastein % of total N	Egg-white % of total N
Amide-N		0·0147	4·1	9·2
Humin-N		0·0055	1·5	2·0
Diamino-N	Total N	0·0756	21·0	22·1
	Amino-N	0·0409	11·3	13·1
	Non-amino-N	0·0347	9·6	8·9
	Arginine-N	0·0404	11·2	11·7
	Histidine-N	0·0066	1·8	0·2
	Lysine-N	0·0286	7·9	10·1
Monoamino-N	Total N	0·2702	74·9	67·6
	Amino-N	0·2670	74·0	66·0
	Non-amino-N	0·0032	0·9	1·6
Total N (Kjeldahl)		0·3608	—	—

The nitrogen distribution of plastein is in general similar to that of its parent substance egg-albumin as determined by Plimmer and Rosedale. The most important differences lie in the values for amide-N and total monoamino-acid-N. Possibly too much stress should not be laid on these differences since, as Gortner and Holm [1917] have shown, the determinations of amide-N are subject to error. If more deamination of monoamino-acids during hydrolysis

of the protein occurred in the experiments of Plimmer and Rosedale than in the case of the hydrolysis of plastein carried out by the author, the differences would be explained.

Nevertheless, support is given to the view that these differences are significant, by comparison with the mean results of analyses of plasteins from various sources carried out by Sawjalow [1908] using the Hausmann-Osborne method and the results obtained by Rosenfeld [1907] by means of the method of Kossel and Kutscher [1900]. These results are given in Table II together with the values for caseinogen after Hart [1901]. They are expressed in percentages of total nitrogen.

Table II.

	Plasteins from various sources (Sawjalow)	Plastein from caseinogen (Rosenfeld)	Plastein from egg-albumin (Folley)	Caseinogen (Hart)	Egg-white (Plimmer and Rosedale)
Amide-N	5·35	3·12	4·1	9·48	9·2
Diamino-N	18·32	20·09	21·0	20·53	22·1
Monoamino-N	76·33	76·79	74·9	69·99	67·6

It can be concluded that on acid hydrolysis plastein yields less amide-N and more monoamino-acid-N than native proteins.

Fractionation of the phenyl iso*cyanate derivative of plastein.*

In the course of an investigation into the nature of serum-albumin peptone it was found by Raper [1907] that well-defined fractions could be separated from the phenyl *iso*cyanate compound of the water-insoluble portion of that fraction of the peptone precipitated by K_2HgI_4. A similar procedure was used in the case of plastein, in view of the possibility that plastein may consist of a mixture of substances of peptone nature.

Preparation of phenylureido-plastein. To a suspension of 10 g. of plastein *A* in 1500 cc. of toluene water were added 20 cc. of 40 % NaOH. After filtering and cooling to 0°, phenyl *iso*cyanate was added in small quantities, each addition being accompanied by vigorous shaking. The temperature of the solution was never allowed to exceed 2°. On the appearance of a white precipitate of diphenylurea, indicating the end of the reaction, the liquid was filtered. The phenyl *iso*cyanate derivative of plastein was precipitated by acidification with acetic acid, the precipitate being filtered off, well washed with cold distilled water and dried *in vacuo*. The finely powdered material was extracted with ether in a Soxhlet apparatus until all traces of diphenylurea had been removed and finally dried for several days in a vacuum desiccator.

A second specimen was prepared from plastein *B* in a similar manner with the difference that in this case a faintly alkaline solution of plastein was used.

Fractionation. A faintly alkaline solution of the sodium salt was prepared by stirring 3 g. of phenylureido-plastein (first preparation) into 300 cc. distilled water, sodium hydroxide being added drop by drop until all solid had

dissolved. Addition of 9 g. of sodium chloride, followed by saturation with carbon dioxide, precipitated a gelatinous substance *A* which was centrifuged out, washed with distilled water and dried *in vacuo*.

From the filtrate a second fraction *B* was precipitated by acidification with acetic acid, this substance being separated in the same manner. The remaining liquid was found to contain very little nitrogen and was not dealt with further.

Substance *A* was boiled for 4½ hours with absolute alcohol and the liquid filtered while hot from the very considerable undissolved residue *C*. After standing in the ice-chest for some days a small quantity of a white powder *D* was deposited from the alcohol. The latter when evaporated to dryness over steam deposited a further small quantity of a white solid *E*. Substances *D* and *E* when dry were of similar appearance—white friable powders—and both melted with blackening over the temperature range 223–228°. It is probable that they were identical.

The substance *C*, which comprised the main portion of the fraction precipitated by carbon dioxide and of the residue *B* precipitable by acetic acid, was titrated with sodium hydroxide in alcoholic solution in presence of phenolphthalein, and, for comparison, the phenyl *iso*cyanate derivative of proteose, the most complex component present in the digests from which the plastein was prepared, was titrated in the same way. The proteose was obtained from a peptic digest of egg-albumin by saturation with sodium sulphate at 37°. The solid was skimmed off and twice purified by solution in warm distilled water, followed by salting out with sodium sulphate. A solution in distilled water was finally dialysed against frequent changes of distilled water for 24 hours. The phenyl *iso*cyanate compound was prepared in the same manner as in the case of plastein.

The equivalent weights obtained were as follows:

Phenylureido-derivative *C*	1025
Phenylureido-derivative *B*	468
Phenylureido-derivative of proteose	782

These results afford no evidence that plastein contains substances of molecular weight of a higher order than that of proteoses.

A similar series of experiments was carried out with precisely similar results, using 3 g. of the second preparation of phenylureido-plastein.

These two experiments indicate that plastein prepared from egg-albumin consists of at least two substances which can be separated by suitable technique.

Summary.

1. The evidence in favour of the view that plastein is a synthetic product is regarded as inconclusive.

2. The molecular weight of plastein was found by the ultracentrifugal method to be 1000 or less.

3. A determination of the nitrogen distribution of plastein shows that this substance contains less amide-N and more monoamino-acid-N than egg-albumin, the protein from which it was derived. The significance of these differences is unknown.

4. The phenyl *iso*cyanate derivative of plastein has been shown to consist of at least two fractions with equivalent weights of the same order as that of proteose. Plastein thus appears to consist of a mixture of substances.

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XIII. IRON IN RELATION TO TYROSINASE.

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By some authors prominent rôles have been ascribed to certain metals in connection with the action of respiratory enzymes. Bertrand [1897] examined preparations of laccase, which he found to contain manganese in proportion to their oxidase activities. He found that addition of a manganese salt to a feebly active preparation increased its activity, and that manganese salts, especially of organic acids, were able to accelerate oxidation of quinol. He concluded that the enzyme laccase consisted essentially of manganese in organic combination, probably with protein. A laccase has, however, been prepared by Suminokura [1930] the action of which was not affected by manganese salts; and the oxidase of Wieland and Fischer [1926] from *Lactarius vellereus*, which had properties similar to those of laccase, contained no manganese.

Iron is another metal which acts as an activator in oxidative processes, and its importance in connection with the action of glutathione has been established by Harrison [1924] and others. Because ferrous salts will bring about an activation of hydrogen peroxide, and because haemoglobin will act as a peroxidase, it has often been thought that peroxidases necessarily contain iron. Willstätter and Pollinger [1923], however, have obtained a preparation of peroxidase which was very active yet contained only 0·04 % of iron; and the activity of their preparations was not proportional to the iron content. Shibata and Shibata [1930] have shown that a number of complex salts of metals such as cobalt, nickel, copper, zinc and cadmium imitate in certain respects the action of oxidases, and have put forward the view that oxidising enzymes are complex compounds containing metals such as these.

The rôle of iron in connection with tyrosinase is specially interesting. The distinctive action of tyrosinase is to convert certain monohydric to *o*-dihydric phenols, since its action on *o*-dihydric phenols can be readily imitated by other enzymic oxidative systems. It has, however, been shown by Martinon [1885] that hydrogen peroxide in conjunction with ferrous salt is able to oxidise phenol to catechol, and Raper (private communication) has now shown that tyrosine itself can be oxidised to 3 : 4-dihydroxyphenylalanine by similar means. Now, hydrogen peroxide is probably produced when tyrosinase brings about oxidation of *o*-dihydric phenols, and it was shown by Pugh [1929] that the action of tyrosinase on monohydric phenols is subject to an initial lag. The

possibility therefore presents itself that the action of tyrosinase preparations on monohydric phenols may be due to the presence of iron as an impurity, or, failing this, that tyrosinase itself contains iron as an essential constituent. Pinhey [1930] has already suggested that tyrosinase necessarily contains iron to account for the inhibition of its action by reagents such as cyanide.

In order to throw more light on these questions, tyrosinase preparations have been made from mealworms, and their properties investigated, using reagents free from detectable quantities of iron. Preparations have also been made from *Lactarius* fungi. The amount of iron in these various preparations has been estimated. The effect of addition of salts of various metals has also been investigated.

Narayanamurti and Ayyar [1929] found that the activity of tyrosinase preparations from *Dolichos lablab*, the field bean, was not decreased by electrodialysis or ultrafiltration, and concluded that the enzyme does not depend for its action on iron or manganese salts. Tyrosinase from potatoes was found by Raper and Wormall [1923], and from mealworms by Pugh [1930], to lose considerably in activity when subjected to ordinary dialysis, but still retained some activity. As part of the present work the cause of loss in activity on ordinary dialysis has therefore been investigated.

Experimental.

Throughout the present work, the following purified reagents were used: freshly glass-distilled water, "iron-free" phosphate buffer as described by Elvehjem [1930], *p*-cresol twice distilled in carbon dioxide, and tyrosine recrystallised twice from glass-distilled water.

Iron estimations.

The estimations of iron were carried out as follows, using the method of Mariott and Wolf [1906] and of Smirk [1928] for the colorimetric estimation. A platinum crucible was used in a first series of experiments, and porcelain in the remainder, without any observable difference. 10 cc. of the enzyme preparation were evaporated to dryness, and ignited to constant weight, 1 cc. nitric acid (50 cc. concentrated nitric acid per litre) was then added, and evaporated to dryness. The residue was dissolved in 1 cc. 10 % HCl, filtered with 5–10 cc. water through a small disc of filter-paper, and made up to a given volume, usually 25 cc. In the later (*Lactarius*) estimations, filtration was omitted, because it was found that ammonium thiocyanate solution caused coloration of even best quality "ashless" filter-paper. The standard iron solution for comparison was made as follows. 0·7 g. ferrous sulphate was dissolved in water, 1·1 cc. pure concentrated sulphuric acid and 0·1 cc. Merck "perhydrol" added, the liquid boiled to remove excess hydrogen peroxide, cooled, and made up to 100 cc. This standard was suitably diluted, usually 1 in 200, for estimations. To 2 cc. liquid to be estimated were added 2 cc. distilled acetone

and 1 cc. 24 % ammonium thiocyanate solution, with shaking and cooling. The colours were compared in a Briggs colorimeter.

Control estimations were carried out, using the usual procedure and reagents, but without any enzyme preparation; filtered through ordinary filter-paper, 0·0044 calculated as mg. Fe per cc. preparation; through "ashless" filter-paper, 0·0059; unfiltered, 0·002.

A preparation of tyrosinase from mealworms was made by the centrifuge method described by Pugh [1930], except that contamination with extraneous iron was carefully avoided, and the mealworms were first of all starved for a few days[1]. Iron was estimated in the preparation both before dialysis and after dialysis for 2 days through a collodion sac into 25 volumes of water, the iron in the outer liquid being also estimated. For each experiment 10 cc. preparation were used.

Whole preparation	0·045;	0·047	mg.	Fe per	cc. preparation
Dialysed residue	0·03;	0·035	,,	,,	,,
Dialysed outer liquid	0·01;	0·01	,,	,,	,,

The outer liquids contained organic matter which had apparently passed through the collodion sac. The preparation had been so well washed that it is improbable that the iron in the outer liquid on dialysis represents altogether inorganic iron salts. The residue, however, definitely contains non-diffusible iron; whether this is a component of organic molecules or is adsorbed inorganic iron is as yet undetermined.

Lactarius rufus fungi (Cannock Chase) were crushed, pressed between porcelain plates, and the juice collected and allowed to stand overnight, after which the sediment was centrifuged off. To 30 cc. juice was added alcohol to 40 %. After a few hours the precipitate was centrifuged off, dissolved in 15 cc. water, and 10 cc. of the solution were taken for iron estimation (*A*). To the alcoholic liquid was added alcohol to 60 %, and the precipitate similarly treated (*B*). Alcohol was then added to 80 % similarly (*C*). Iron was also estimated in the final 80 % alcoholic liquid, and in whole juice. Preparation of *A* was carried out a second time.

Whole juice ...	0·021	mg.	Fe per	cc. juice
A	0·0031	,,	,,	,,
B	0·0044	,,	,,	,,
C	0·0096	,,	,,	,,
Alcoholic liquid	0·010	,,	,,	,,
Second *A* ...	0·0028	,,	,,	,,

Bertrand and Muttermilch [1907] showed that tyrosinase acts on tyrosine but not on guaiacol, while laccase acts on guaiacol but not on tyrosine. The tyrosinase and laccase activities of the *Lactarius* preparations were therefore determined by noting the time required for first appearance of colour when

[1] Note on starved mealworms. The action on catechol of a preparation made from starved mealworms was found to be retarded and never accelerated by hydrogen peroxide. The slight initial acceleration noted by Pugh [1930] was apparently due to a trace of peroxidase, which is present in the foodstuff. Acceleration of action on tyrosine and *p*-cresol was unaltered.

0·5 cc. *A*, *B* or *C* was added to (1) 2 cc. phosphate buffer p_H 6·5, (2) 2 cc. saturated tyrosine in phosphate buffer p_H 6·5, (3) 2 cc. 0·2 % guaiacol in phosphate buffer p_H 6·5.

	A	*B*	*C*
(2)	10 mins.	20 mins.	Slight next day
(3)	4 „	Slight next day	Slight next day

The amount of iron in these *Lactarius* preparations is thus very small, and no direct relationship to oxidase activity is apparent.

Lactarius rufus fungi (Delamere) and *Lactarius turpis* fungi (Delamere) were treated similarly, and similar iron estimations carried out. 40 cc. *rufus* juice were used, 60 cc. *turpis* juice. A fresh set of reagents was used, and a fresh control estimation carried out, which yielded the figure 0·0005.

L. rufus		*L. turpis*	
Whole juice ...	0·010 mg. Fe per cc. juice	Whole juice ...	0·012 mg. Fe per cc. juice
A	0·005 „ „ „	*A*	0·006 „ „ „
B	0·0025 „ „ „	*B*	0·003 „ „ „
C	0·0025 „ „ „	*C*	0·005 „ „ „
Alcoholic liquid	0·003 „ „ „	Alcoholic liquid	0·005 „ „ „

The tyrosinase and laccase activities of the preparations were determined as before.

L. rufus:	Whole juice	*A*	*B*	*C*
(2)	3 mins.	7 mins.	5 mins.	Overnight
(3)	At once	1 min.	8 mins.	25 mins.
L. turpis:				
(2)	At once	3 mins.	50 mins.	Overnight
(3)	25 mins.	Overnight	Nil in two days	Nil in two days

Note. Whole juice was half as concentrated as the other preparations.

Again no relationship between iron content and oxidase activity is apparent, and it is evident that the oxidases occur in the juices contaminated with impurities which do contain iron.

In order to calculate the percentage content of iron, 10 cc. of each of these preparations were evaporated to dryness on the water-bath, dried in an oven at 110°, and the dry residue weighed. The smallest percentage content of iron was that in the alcoholic liquid residues, which contained only 0·01 % of iron. The smallness of this percentage is accounted for by the presence of substances which neither contain iron nor are precipitated by alcohol. The smallest percentage in an active tyrosinase preparation was 0·07 % in *L. turpis A*.

Effect of metallic salts.

The precautions taken to avoid contamination with extraneous iron in no wise prevented tyrosinase preparations from mealworms or fungi from acting on the monohydric phenols, tyrosine and *p*-cresol. Also, action on these substrates was still hastened by hydrogen peroxide. With precautions to avoid iron, however, the coloration of tyrosine and of *p*-cresol solutions by hydrogen

peroxide without enzyme reported by Pugh [1930] was observed only after many days with *p*-cresol and not at all with tyrosine, and must therefore have been due to contamination with iron salts.

The experiments next to be described were carried out to find the effect of addition of metallic salts on the action of tyrosinase on monohydric phenols in buffered solution. The coloration of tyrosine and of *p*-cresol by tyrosinase was hastened by addition of either ferrous salt or hydrogen peroxide in small quantities. With *p*-cresol, however, the colour in presence of iron was yellow, in presence of hydrogen peroxide, pink. The tyrosine tubes all acquired first of all a reddish colour, but later, when blackening occurred, those containing iron became more blackish than those without it; this is probably due to the direct action of the iron salt on the *o*-dihydric phenol. In each series tube 1 contained 2 cc. phosphate buffer p_H 7·0. In the first series tubes 2–26 contained 2 cc. saturated tyrosine solution in phosphate buffer p_H 7·0. In the second series tubes 2–26 contained 2 cc. 0·2 % *p*-cresol solution in phosphate buffer p_H 7·0. Hydrogen peroxide, in the form of Merck "perhydrol," was added as stated in the following Table, and iron in the form of ferrous ammonium sulphate solution. 0·5 cc. tyrosinase from mealworms, dialysed into distilled water, was added, diluted 1 in 10 for action on *p*-cresol. The contents of each tube were made up to 3 cc. with water. The time of onset of coloration was noted.

Tubes	0·5 cc. "perhydrol" %	Mg. Fe	First series (tyrosine). Time	Second series (*p*-cresol). Time
2	Nil	Nil	6 hrs.	About 1 hr.
3	0·5	,,	15 mins.	<5 mins.
4	0·05	,,	2 hrs.	15–20 ,,
5	0·005	,,	4 ,,	30 ,,
6	0·0005	,,	6 ,,	Nearly 1 hour
7	Nil	0·5	25 mins.	10 mins.
8	,,	0·05	3 hrs.	25 ,,
9	,,	0·005	5 ,,	40 ,,
10	,,	0·0005	6 ,,	Nearly 1 hr.
11	0·5	0·05	15 mins.	<5 mins.
12	0·05	,,	2 hrs.	15–20 ,,
13	0·005	,,	3 ,,	20 ,,
14	0·0005	,,	3 ,,	15 ,,
15	0·5	0·005	15 mins.	<5 ,,
16	0·05	,,	2 hrs.	15–20 ,,
17	0·005	,,	4 ,,	30 ,
18	0·0005	,,	5 ,,	40 ,,
19	0·05	0·5	17 mins.	5–10 ,,
20	,,	0·05	2 hrs.	15 ,,
21	,,	0·005	2 ,,	15 ,,
22	,,	0·0005	2 ,,	15 ,,
23	0·005	0·5	20 mins.	10 ,,
24	,,	0·05	3 hrs.	20 ,,
25	,,	0·005	4 ,,	30 ,,
26	,,	0·0005	4 ,,	30 ,,

As controls both series were repeated with omission of tyrosinase, and tubes in which colour appeared were repeated also without either tyrosine or *p*-cresol. In tubes 19 and 23 yellow colour appeared within 5 minutes, even in absence of tyrosine or *p*-cresol, and was evidently due to oxidation of ferrous to ferric

iron by hydrogen peroxide. After three days colour had appeared only in control tubes 11, 12, 15, 19, 20 and 23, which were very slightly greyish in series I, and slightly yellowish-pink in series II.

Salts tested in this way were (besides ferrous ammonium sulphate) ferrous sulphate, manganese sulphate, copper sulphate, cobalt chloride, calcium chloride, zinc chloride and cadmium chloride. The quantities used were approximately equimolecular with the quantities of ferrous ammonium sulphate stated above. Only the iron salts affected the rate of coloration of tyrosine and *p*-cresol by tyrosinase, with the possible doubtful exceptions of copper and cobalt. Ferrous sulphate in larger quantity (1 mg.), as used by Gortner [1910] and by Onslow [1917], was found to retard the action of tyrosinase, as reported by them.

Addition of the above salts at a dilution of about $M/5000$ to the bottles of Barcroft micro-respirometers in which tyrosinase was acting on tyrosine, *p*-cresol, or catechol, had no significant effect on the oxygen absorptions. The following mixtures were placed in the bottles of the first respirometer. Left: 1 cc. saturated tyrosine solution, or 0·5 % *p*-cresol or catechol solution in phosphate buffer p_H 6·5; 1 cc. buffer; 0·5 cc. water; 0·5 cc. tyrosinase from mealworms (dialysed against phosphate buffer p_H 8·0) diluted 1 in 8 for action on *p*-cresol and 1 in 20 for action on catechol. Right: 2 cc. buffer; 0·5 cc. water; 0·5 cc. enzyme preparation. In the other respirometers, 0·5 cc. water was replaced by 0·5 cc. metallic salt solution in both bottles. To each bottle a little solid thymol was added. Concentrated caustic potash was placed in the small tube fused into each bottle.

		H_2O	Fe	Cu	Co	Mn	Ca
Tyrosine.	mm.³ O_2 absorbed after 1 hr.	36	47	33	26	29	25
p-Cresol.	,, ,, ,, ,, ½ hr.	249	221	203	223	233	205
Catechol.	,, ,, ,, ,, 1 hr.	177	183	153	156	182	152

The effect of the ferrous salt was evidently too small to be measured by these means.

The effect of addition of metallic salts was also investigated using tyrosinase prepared from *L. turpis* juice as follows. The juice was treated with alcohol to 40 % and centrifuged; after addition of alcohol to 60 % the liquid was again centrifuged, and this last residue was dissolved in water to half the volume of the original juice. The juice acted not only on tyrosine but also quickly on guaiacol. The tyrosinase preparation did not act on guaiacol even after many days. In the following tests, tube 1 in every series contained 2 cc. phosphate buffer p_H 6·5. In series I and II tubes 2–10 contained 2 cc. saturated tyrosine solution in phosphate buffer p_H 6·5. In series III and IV tubes 2–10 contained 2 cc. 0·2 % *p*-cresol solution in phosphate buffer p_H 6·5. In series V tubes 1–10 contained 2 cc. phosphate buffer. Hydrogen peroxide and iron (as $FeSO_4$) were added to each series as stated in the following Table. 0·5 cc. enzyme preparation was added to series I and III, diluted 1 in 3 for

action on *p*-cresol. The contents of each tube were made up to 3 cc. with water. The time required for commencement of coloration was noted.

Tubes	0·5 cc. "perhydrol" %	Mg. Fe	Series I (mins.)	Series III (mins.)
2	Nil	Nil	70	35
3	0·05	,,	12	10
4	0·005	,,	25	20
5	Nil	0·05	15	8
6	,,	0·005	16	10
7	0·05	0·05	10	5
8	,,	0·005	12	8
9	0·005	0·05	12	8
10	,,	0·005	13	10

Next day, in series II, IV and V, tubes 5, 7 and 9 were yellowish. In series IV, in addition, tubes 7 and 8 were pinkish.

Tubes 5–10 of all the above series were also repeated, using *A* manganese sulphate, *B* copper sulphate, *C* cobalt chloride, in place of ferrous sulphate, quantities being approximately equimolecular with the quantities of ferrous sulphate stated above.

Tubes	*A* I (mins.)	*A* III (mins.)	*B* I (mins.)	*B* III (mins.)	*C* I (mins.)	*C* III (mins.)
5	80	35	35	35	75	35
6	90	35	70	35	70	35
7	12	10	30	12	8	10
8	12	12	12	15	10	20
9	25	20	30	23	25	25
10	25	20	20	25	25	25

The colour produced in series *B* I tubes 5, 6, 7 and 9 was not reddish but greenish-grey. In series *B* II, IV and V, tubes 5 and 9 were bluish, and, in addition, in series II tube 7 became grey; series IV tube 7 orange-pink, tube 8 pink; series V tube 7 was bluish. No essential difference was noted between the use of tyrosinase from mealworms and from fungi.

Using as metallic salts ferrous sulphate and manganese sulphate, series I and III were also repeated with crude *L. turpis* juice in place of tyrosinase preparation.

Tubes	Iron I (mins.)	Iron III (mins.)	Manganese I (mins.)	Manganese III (mins.)
2	30	35	—	—
3	4	5	—	—
4	10	15	—	—
5	5	5	30	35
6	8	8	33	35
7	1	3	4	5
8	6	5	4	5
9	1½	4	10	15
10	7	6	10	15

Although the first appearance of colour was not hastened, the colour produced from *p*-cresol by the crude fungus juice deepened more rapidly in presence of manganese salt than in its absence; this may possibly be connected with the presence of laccase in the crude juice.

The coloration of tyrosine and of *p*-cresol by hydrogen peroxide with ferrous salt was evident in the experiments described above. The following tests were carried out to find the smallest amount of ferrous salt necessary to make this reaction apparent. In one series of test-tubes were placed 2 cc. water, in another series 2 cc. saturated tyrosine in water. To the first tube of either series was added 0·5 cc. containing 0·5 mg. Fe in the form of ferrous sulphate, to the second pair 0·05 mg. Fe, to the third pair 0·005 mg. Fe, and so on, finally a pair with water in place of iron salt solution. To each tube was added 0·25 cc. 1 % hydrogen peroxide. The first three tubes without tyrosine showed a yellow colour owing to the action of hydrogen peroxide on the ferrous salt. The first and second tyrosine tubes became rapidly green, then brown. The green colour first produced is evidently due to the reaction of *o*-dihydric phenol with ferric salt. The third tyrosine tube became pinkish, later brown; this corresponds to an added iron content of 0·005 mg. Effect in further tubes was not immediate, but after several days effect was detectable as far as an added iron content of 0·00005 mg. The above brown colours in presence of hydrogen peroxide tended to fade. The above tests carried out on tyrosine in phosphate buffer p_H 6·5 instead of aqueous solution of tyrosine yielded a detectable effect only to an added iron content of 0·005 mg., iron being precipitated as phosphate.

Attempts were made to imitate the action of tyrosinase by means of mixtures of peroxidase from horse-radish with hydrogen peroxide and ferrous salt. To 2 cc. saturated tyrosine in phosphate buffer p_H 7·0 were added 1 cc. 0·05 % hydrogen peroxide, 2 cc. peroxidase preparation, and 1 cc. ferrous sulphate solution containing 0·1 mg. Fe. A comparison tube contained buffer in place of tyrosine solution; in this a slight yellow colour appeared. The liquid in the tyrosine tube became slightly yellow, then pink, but the pink colour faded, and no darkening was noticeable. Variations were made in the quantities used, and aqueous solution was tried in place of buffer, but darkening was observed only when it took place likewise in absence of peroxidase. Continuous catalytic action, such as is shown by tyrosinase, was not successfully imitated. In the preceding experiment the iron salt solution was also replaced by 0·5 cc. 0·002 % catechol in buffer. A deep pink colour appeared, which faded without darkening. This pink colour is evidently due to the reaction shown by Pugh [1930] to occur between tyrosine and *ortho*quinone, produced from catechol by peroxidase with hydrogen peroxide.

A few observations have been made concerning the action of iron salts on certain phenols. It is well known that with dilute ferric chloride solution phenol or *p*-cresol gives a violet colour and *o*-dihydric phenols give a green colour followed by darkening. If, however, a drop of 1 % hydrogen peroxide is added to the violet liquid, after a few seconds' delay it becomes green. This appears to indicate that not only ferrous but also ferric iron may be able to bring about the oxidation of these monohydric phenols to *o*-dihydric phenols by hydrogen peroxide. Ferrous sulphate solution and hydrogen peroxide added to phenol or *p*-cresol solution produce a green colour forthwith. Ferrous iron is converted

into ferric iron by hydrogen peroxide, but ferric chloride alone does not oxidise phenol or *p*-cresol to *o*-dihydric phenol, since solutions of phenol or *p*-cresol with ferric chloride are violet, and do not become green when left to stand. Ferrous sulphate and hydrogen peroxide added to guaiacol solution in water produce a green colour followed by darkening.

Loss in activity on dialysis.

The loss in activity of tyrosinase from mealworms on dialysis was investigated as follows. Dialysis in a collodion sac was tried first into ammoniacal water; this lost in alkalinity, and the preparation lost in activity, even when its alkalinity was restored. Dialysis was then tried into dilute sodium hydroxide solution, p_H 8·0; this also lost in alkalinity, presumably owing to the buffering action of the proteins in the preparation. Dialysis was next tried into phosphate buffer of various p_H values. At p_H 8·5 there was no appreciable loss in activity on dialysis. In these experiments the ammoniacal extract of tyrosinase from mealworms, prepared by the centrifuge method, was centrifuged, and 10 cc. were dialysed into 500 cc. phosphate buffer at *A* p_H 7·0, *B* p_H 8·0, *C* p_H 8·5; *D* was kept undialysed. After two days each was made up to 12 cc. and centrifuged. There was much precipitate in *A*. Each was diluted 1 in 10 for action on *p*-cresol, 1 in 100 for action on catechol. 0·5 cc. was added to 2 cc. 0·2 % *p*-cresol or catechol at p_H 6·5, and to buffer for comparison. Times required for appearance of colour were as follows:

A	*p*-Cresol	10 mins.	Catechol	1 min.
B	,,	5 ,,	,,	½ ,,
C	,,	4 ,,	,,	20 secs.
D	,,	3½ ,,	,,	20 ,,

Loss in activity on dialysis is therefore probably due to some irreversible change resulting from loss in alkalinity.

The presence of phosphate was found not to affect the time required for appearance of colour by the action of tyrosinase on its substrates, but it did affect the final intensity of colour. The following mixtures were placed in test-tubes (1) 4 cc. water, (2) 2 cc. water and 2 cc. phosphate buffer of the same p_H as the water, (3) 2 cc. water and 2 cc. 0·2 % *p*-cresol in water, (4) 2 cc. water and 2 cc. 0·2 % *p*-cresol in the same phosphate buffer. To each was added 0·5 cc. tyrosinase from mealworms dialysed into water made faintly alkaline with ammonia, then centrifuged. In both (3) and (4) a yellow colour appeared after 18 mins. When *p*-cresol was replaced by catechol, the enzyme preparation being diluted 1 in 10, colour appeared in both (3) and (4) after 3 mins. Next day, however, coloration was much further advanced in the tubes containing phosphate buffer than in those without it. This is apparently due to the maintenance of a p_H near the optimum for tyrosinase by the buffering action of the phosphate.

Discussion.

An attempt has been made to discover whether the action of tyrosinase depends in any way on the presence of iron. The iron estimations described in this paper have not revealed any relationship between the iron content and the oxidase activity of various preparations of tyrosinase. They therefore provide no evidence that tyrosinase necessarily contains iron. Tyrosinase in the fungus juices used was shown to be contaminated with impurities which do contain iron. If the smallest iron content found in an active tyrosinase preparation is used as a basis to calculate the minimum molecular weight of tyrosinase, on the assumption that each molecule contains one atom of iron, the result, about 100,000, is by no means more than is to be expected. It was shown that a tyrosinase preparation from mealworms contains non-diffusible iron. This may or may not be part of the tyrosinase. There is therefore no evidence that tyrosinase does not necessarily contain iron. The limits of direct estimability of iron have been approached, and it is evident that direct estimation of iron in tyrosinase preparations will not provide an answer to the problem.

Small quantities of ferrous salts hasten the appearance of colour in mixtures of tyrosinase with the monohydric phenols, tyrosine or *p*-cresol, although larger quantities inhibit the action of the enzyme. Since hydrogen peroxide with ferrous salt is able to oxidise certain monohydric to *o*-dihydric phenols, it is reasonable to suppose that the activating effect of ferrous salts on the action of tyrosinase depends on this reaction. This would involve the assumption that hydrogen peroxide is produced by the action of tyrosinase on its substrate, which is very probable; the apparent hastening of the action of tyrosinase on monohydric phenols by ferrous salts, in fact, appears to be indirect evidence of the production of hydrogen peroxide.

The essential action of tyrosinase is to bring about the oxidation of certain monohydric to the corresponding *o*-dihydric phenols, and this action is hastened by some product or products of action of tyrosinase. Nevertheless, the fact that hydrogen peroxide with ferrous salt is able to imitate the characteristic action of tyrosinase is no real evidence that tyrosinase contains iron. The action of this reagent (Fenton's reagent) as an oxidising agent is well known. It is less specific than the action of tyrosinase. In particular, tyrosinase does not cause oxidation of guaiacol, but there is indication that guaiacol may be oxidised by hydrogen peroxide with ferrous salt to an *o*-dihydric phenol.

It is interesting to compare the smallest concentration of iron which, as ferrous salt with hydrogen peroxide, will bring about immediate coloration of tyrosine with the amount of iron in a similar tyrosinase preparation. The smallest effective concentration of inorganic iron was found to be about 0·002 mg. per cc., which is of the same order as the amount of iron in a similarly effective tyrosinase preparation, *L. turpis A*, containing the smallest percentage of iron of any of the present preparations. More highly purified preparations might contain less iron, since the crude tyrosinase is associated

with impurities which contain iron, but on the other hand it is probable, as Shibata and others have suggested, that the activity of iron in appropriate organic combination would be greater than that of inorganic iron. It is by no means impossible that the activity of tyrosinase is due to iron in the tyrosinase molecule. It is evident, however, that other methods must be devised before the essential nature of tyrosinase can be elucidated.

The effect of iron in causing coloration of phenols has been known for many years. Walter [1898] showed that pure solid phenol remains uncoloured for months in paraffin-coated vessels; whereas addition of a little ferrous sulphate produced coloration in three days, and of hydrogen peroxide in addition, in two days. The action of iron without hydrogen peroxide was ascribed to the formation of hydrogen peroxide from air and moisture by the iron.

The mechanism by which iron salts bring about oxidations by means of hydrogen peroxide has been a subject of some speculation. Although hydrogen peroxide oxidises ferrous to ferric salt, ferric salt alone does not bring about oxidation of monohydric phenols. Some indication is, however, given in this paper that both ferrous and ferric iron will catalyse the oxidation of monohydric phenols by hydrogen peroxide. This would be in agreement with the theory put forward by Mummery [1913] of the oxidising action of iron salts with hydrogen peroxide. He supposed that hydrogen peroxide forms with iron salts a ferrous perhydrol formulated FeX(O.OH), or a ferric perhydrol FeX_2(O.OH), both of these being able to act as oxidising agents, with reformation of ferrous or ferric salt; the former, however, being much the more effective oxidising agent.

Inorganic iron salts do not appear to play any essential part in the ordinary action of tyrosinase. The loss in activity of tyrosinase preparations on ordinary dialysis can be prevented by carrying out the dialysis into alkaline buffer solution, and is therefore due apparently to some irreversible change resulting from loss in alkalinity, and not to loss in iron or any other specific salts.

Summary.

1. The iron contents of some tyrosinase preparations from mealworms and from fungi have been estimated. Results of such estimations, however, can provide no conclusive evidence with regard to the nature of tyrosinase.

2. The coloration of tyrosine and of *p*-cresol solutions with tyrosinase is hastened by ferrous salts in small quantity. This provides indirect evidence that hydrogen peroxide is produced during the action of tyrosinase.

3. The oxidation of monohydric to *o*-dihydric phenols by hydrogen peroxide with ferrous salt is less specific than the action of tyrosinase, and provides only a very imperfect imitation of the action of tyrosinase.

4. Means must be devised along other lines to determine whether tyrosinase contains iron or not.

5. The loss in activity of tyrosinase from mealworms on dialysis may be

prevented by carrying out the dialysis into alkaline buffer solution, and is therefore not due to loss of iron or other specific salts.

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XIV. THE INFLUENCE OF THE GONADS ON PROTEIN METABOLISM.

I. URINARY CREATININE AFTER CASTRATION.

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In the first of a series of papers on the sex glands and metabolism, Korenchevsky [1925] summarises the results of castration and ovariectomy on obesity and on gaseous and nitrogen metabolisms obtained by various investigators since 1890. In regard to nitrogen metabolism some observers report no effect, some a decrease and others an increase after gonadectomy. Luthje [1902] for instance was unable to find any change in the nitrogen balance of a castrated dog. Korenchevsky's own work on dogs and rabbits showed that removal of the gonads leads to a decrease in the total daily urinary nitrogen in fed animals. This effect was more pronounced in fasting animals. The only reference to the influence of castration on creatine or creatinine metabolism is a paper by Tsun-Chee-Shen [1927] who reported an unchanged creatine metabolism after castration in dogs and albino rats. Werner [1929] found an increase in the blood-urea of castrated sheep. Most workers confined themselves to an investigation of the changes in nitrogen balance or nitrogen output. In the following research the effect on urinary creatine and creatinine was investigated because of the obvious fact that these end-products are much more accurate indices to changes in endogenous protein metabolism than the total nitrogen excretion.

The dates corresponding to the points on the graph refer to the final 24-hour period of each series. The arithmetical mean of the daily creatinine outputs of each series is plotted.

Experimental.

The experiments were performed on adult male rabbits. The animals were kept in metabolism cages, fed on cabbage leaves and carrots, and the urine was collected every 24 hours. From the Tables, it can be seen that the daily output of creatinine was very constant. This indicated that complete 24-hour urines had been collected and consequently catheterisation, *etc.* was considered unnecessary. At the end of each 24-hour period the sides and funnel-shaped bottom of the collecting portion of the cage were washed with water and the washings added to the urine. The clear filtrate from about 100 cc. of

the diluted urine was used for the estimations of creatine and creatinine by Folin's method. The colorimetric readings were usually checked independently by one or other of us.

The animals remained in the cages for 3–8 days; in some cases for a longer period. They were then put into a large cage which communicated with a large open run on the outside of the animal house and were fed on bran, oats and vegetables. After 1–4 weeks they were returned to the metabolism cages. Both when in the metabolism cages and when in the general run the animals got plenty of sunlight, and throughout the course of the experiment (several months) they were in a healthy condition.

Collection of urine from operated animals was usually begun about a fortnight after the operation. The testis for grafting was taken from a rabbit, divided into two, and each half inserted between the external and internal oblique muscles, one on either side just above and lateral to the symphysis pubis. In those cases in which the graft was subsequently removed it was found that the transplanted testis had become vascularised.

Experimental data.

In the following Tables the dates refer to the first of a number of 24-hour periods. The arithmetical mean and standard deviation of the daily creatinine output in milligrams are given for each series of 24-hour periods.

Table I.

Date		Daily urinary creatinine mg.	No. of 24-hr. periods	Weight kg.
A. Rabbit No. 1. Castrated 17. ix. 29. Grafted 2. v. 30.				
5. ix. 29	Pre-period	85 ±5·0	12	2·03
8. x. 29	After castration	90 ±1·4	3	2·25
30. x. 29	,,	92 ±2·3	5	2·33
20. xi. 29	,,	97 ±2·0	4	2·46
10. i. 30	,,	105 ±2·2	4	2·55
1. ii. 30	,,	104 ±4·5	5	2·55
17. ii. 30	,,	108 ±6·0	5	2·62
19. iii. 30	,,	110 ±7·2	5	2·63
14. iv. 30	,,	109 ±5·2	6	2·79
19. v. 30	After grafting	106 ±6·1	3	2·68
2. vi. 30	,,	101 ±8·1	3	2·61
16. vi. 30	,,	92 ±7·0	3	2·66
B. Rabbit No. 2. Castrated 19. xii. 30. Grafted 23. ii. 31.				
10. xii. 30	Pre-period	61 ±6·5	7	1·94
5. i. 31	After castration	57 ±5·7	7	2·25
20. i. 31	,,	80 ±7·7	8	2·23
9. ii. 31	,,	84 ±6·7	8	2·27
2. iii. 31	After grafting	61 ±5·0	4	2·30

These two preliminary experiments (Table I *A* and *B*) show that removal of the testes results in a definite increase in the daily output of creatinine. In the case of rabbit No. 2 the daily urinary creatinine had risen, a month after the operation, from 61 mg. to 80 mg., a month later this high level was still

maintained, the mean being 84 mg. A fortnight after grafting, the creatinine excretion had fallen to the normal pre-castration value of 61 mg. Similar results were obtained for rabbit No. 1. Seven months after castration the urinary creatinine was still 25 % above the value before the operation.

Table II.

Date		Daily urinary creatinine mg. Operated animal	Daily urinary creatinine mg. Control animal	No. of 24-hr. periods	Weight kg. Operated	Weight kg. Control
Rabbit No. 3. Castrated 23. ii. 31. Grafted 22. vii. 31. Rabbit No. 4 (Control).						
8. ii. 31	Pre-period	60 ± 1·9	58 ± 4·4	8	2·05	1·87
4. iii. 31	After castration	62 ± 6·0	61 ± 5·0	6	2·02	1·81
13. iv. 31	,,	75 ± 5·3	61 ± 2·1	5	1·95	1·81
10. v. 31	,,	79 ± 1·3	64 ± 0·4	5	2·0	1·77
13. vii. 31	,,	89 ± 8·0	Died	6	1·94	—
27. vii. 31	After grafting	77 ± 6·2	—	3	1·84	—
10. viii. 31	,,	63 ± 6·4	—	5	1·88	—
18. viii. 31	,,	59 ± 3·7	—	5	1·88	—

Animal No. 3 (Table II) shows a 50 % increase five months after castration, the normal control having remained at a steady level during that period. About three weeks after grafting the creatinine excretion was again normal.

The experiment in the case of rabbit No. 5 was carried further and the graft removed after its effect had become fully established. This resulted, about a fortnight after removal, in the reappearance of the usual castration effect. The complete experiment is graphically represented in Fig. 1.

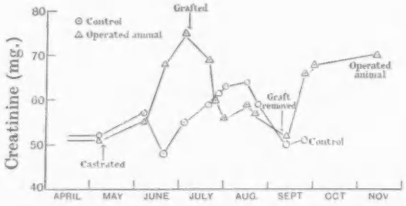

Fig. 1. The dates corresponding to the points on the graph refer to the final 24-hour period of each series. The arithmetical mean of the daily creatinine outputs of each series is plotted.

Read [1921] in a study of the metabolism of eunuchs found that castration before puberty induced a continuance into adult life of the pre-pubertal creatinuria, but castration after puberty did not result in excretion of creatine. Tsun-Chee-Shen and Hao Lin [1927] report that creatinuria was present in only one case out of nine eunuchs examined by them. No creatine was found in the urines of the above castrated adult rabbits, thus confirming the results in eunuchs.

SUMMARY.

1. Castration of adult male rabbits gives rise to a 25–50 % increase in the excretion of urinary creatinine.

2. Grafting of testes into castrated rabbits causes a fall in the high creatinine excretion to the normal pre-castration level.

3. Subsequent removal of the graft leads to the reappearance of the usual castration effect—increased creatinine output.

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XV. THE ESTIMATION OF GLUTAMINE IN THE PRESENCE OF ASPARAGINE.

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In connection with a research into the nitrogenous constituents of forage grasses which we are undertaking in this laboratory we have investigated the different methods used by plant chemists for determining the various groups of nitrogen present in the aqueous extract. It was necessary to find out whether the determination of amino-N by the Van Slyke method should be carried out (1) direct, (2) after removing ammonia and volatile bases by distillation with magnesia, or (3) after mild hydrolysis [Sachsse, 1873] to hydrolyse acid amides and subsequent removal of ammonia with magnesia. In most grass extracts very little difference was found between (1) and (2), but with other green leaves, as for instance, those of young sunflower or castor-oil plants there was a fall in amino-N after removal of ammonia which was difficult to account for. Ammonia itself gives only about $\frac{1}{3}$ of its N after ten minutes in the Van Slyke apparatus, and the fall observed was many times the total ammonia-N value. It seemed to us that the anomaly might be due to glutamine, and we accordingly investigated the behaviour of this substance under similar conditions.

We were surprised to find that glutamine gave nearly the whole of its N in the Van Slyke apparatus, that is, not only did the amino group react normally with nitrous acid, but the amide group also reacted. A search of the literature showed that this abnormal behaviour was noticed by Thierfelder and von Cramm [1919] and also by Schulze and Bosshard [1883], who showed that whereas asparagine gave half its N as "amido-N" by Sachsse and Kormann's [1875] method (based on the reaction between the modern "amino-groups" and nitrous acid), glutamine gave the whole of it. As far as we are aware these two observations have remained unnoticed by those plant chemists who have employed the Van Slyke method for determining amino-N.

It was shown by Bosshard [1883] that glutamine was much less stable than asparagine, and that considerable hydrolysis of the amide group occurred on boiling with magnesia. For this reason Schulze [1906] recommended that the

free ammonia-N of plant extracts should be determined by distillation with magnesia *in vacuo* at 40°. As the removal of ammonia in steam in the ordinary micro-Kjeldahl apparatus takes under 10 minutes we investigated the percentage breakdown of glutamine when distilled with magnesia at 100° in this apparatus. Ammonia representing about 27 % of the amide groups was obtained in 10 minutes. The residue from the distillation flask was withdrawn, made just acid with acetic acid, concentrated, and the amino-N determined by Van Slyke's method. To our surprise this had fallen to 28 % of the total N. It was clear that further hydrolysis of the amide group had occurred during the concentration in weak acid solution, but even so the conversion of the resulting glutamic acid into pyrrolidonecarboxylic acid—if that were the explanation of the large decrease in amino-N—had taken place much more rapidly than Foreman's [1914] researches on this transformation would suggest.

It seemed to us that the relative instability of glutamine compared with asparagine might be made the basis of a method for the separate determination of these two substances in plant extracts; we accordingly undertook a systematic investigation of the stability of glutamine and certain other substances at various p_H values, measuring both the amount of ammonia formed and the fall in amino-N under controlled conditions.

Experimental.

Estimation of ammonia- and amino-N.

The ordinary micro-Kjeldahl apparatus was used to determine ammonia in steam at 100°. For the determination *in vacuo* at 40° the micro-apparatus of Parnas and Heller [1924] was employed. The flask was made detachable to permit the withdrawal of the residual liquid after distillation with as little dilution as possible.

The procedure was as follows. 10 cc. of the solution or plant extract were washed into the flask, which was then connected to the apparatus. 3 cc. of a 10 % suspension of magnesia were then run in through the special burette provided for this purpose. The ammonia was removed by distilling for 15 minutes at a pressure of about 3 cm., the flask being immersed in a water-bath at 40°. A fine stream of air was drawn through the apparatus instead of the steam employed by Parnas and Heller, as this allowed a higher vacuum to be maintained without continuous watching and also concentrated the liquid in the distilling flask to a volume of about 5 cc. 8–10 cc. of distillate were collected. Control experiments with ammonium sulphate showed that 98 % of the ammonia passed over under these conditions. At the end of the operation the flask was detached from the apparatus and sufficient acetic acid was added to the residual liquid to dissolve the excess of magnesia. The solution was then carefully washed into a graduated 10 cc. flask and amino-N determined on 2 cc. aliquots in the Van Slyke micro-apparatus.

The relative stability of glutamine and asparagine.

The results of a series of experiments with 30 mg. samples of glutamine and asparagine are given in Table I. It will be seen that a reaction time of 10 minutes in the Van Slyke apparatus gives only 49·5 % of the total N of asparagine, but 92 % of that of glutamine.

Table I. *Showing the relative stability of asparagine and glutamine.*

(Values given are percentages of total N.)

	Amide-N				Time of shaking in Van Slyke apparatus (mins.)	Amino-N	
Substance	Whether magnesia used	Temperature	Time of distillation (mins.)	Amide-N found		Found	Theory
Glutamine	—	—	—	Not determined	10	92	50
Glutamine	—	—	—	,,	30*	94	50
Glutamine	—	—	—	,,	150*	97·5	50
Asparagine	—	—	—	,,	10	49·5	50
Glutamine	No	100°	5	8·9	10	78	50
Glutamine	,,	40°	15	1	10	92	50
Glutamine	Yes	100°	10	13·5	10	74	50
Glutamine	,,	40°	15	1	10	86	50
Asparagine	,,	100°	10	1·4	10	50	50
Asparagine	,,	40°	15	0·3	10	50	50
Urea	,,	40°	15	0	—	10	No amino-N
Allantoin	,,	40°	15	0	—	1·2	,,

* Intermittent shaking.

It was shown by Plimmer [1925] that the amide groups of asparagine and of other amides such as acetamide and propionamide were stable to nitrous acid in acetic acid solution, but that they reacted completely in 24 hours in strong HCl solution. He suggested that in aqueous or acetic acid solution the amides had the formula R.C(OH) : NH, which was converted into the tautomeric $R.CO.NH_2$ by mineral acid. They could then react with nitrous acid in virtue of the $—NH_2$ group. If this is so then glutamine appears to be the only amide possessing the $R.CO.NH_2$ structure in aqueous or acetic acid solution. When an aqueous solution of glutamine was boiled at 100° for 5 minutes 17·8 % of the amide-N was split off. This value was increased to 27 % in the presence of magnesia. In each case there was a corresponding fall in amino-N. Boiling an aqueous solution *in vacuo* at 40° for 15 minutes caused no decomposition and in the presence of magnesia there was only a small liberation of ammonia, equal to 2 % of the amide-N, and a fall in amino-N of 6 %, suggesting a small amount of anhydride formation. These results for amide-N confirm those of Schulze [1906]. In all subsequent experiments described in this paper amide-N was determined by distillation *in vacuo* with magnesia for 15 minutes, and in the case of glutamine the control value for amide-N was taken as 1 % and of the amino-N as 86 % of the total N.

We next proceeded to a more comprehensive series of experiments to

determine the stability at various p_H values. The procedure was as follows. 30 mg. samples of glutamine, asparagine and glutamic acid were placed in boiling-tubes and 10 cc. of the requisite buffer solution added. The tubes were then heated in a boiling water-bath for the required time, cooled and the contents transferred to the flask of the amide-N apparatus. Amide-N and amino-N were determined. The results are given in Table II.

It will be seen that asparagine is quite stable over the range p_H 4–8, and that the amide-N is only liberated to the extent of 7 % at p_H 2. Glutamine, however, is rapidly decomposed, and relatively large amounts of amide-N are given at p_H 3, 4, 5, 8, 9 and 10. Glutamic acid undergoes transformation to pyrrolidonecarboxylic acid at about the same rate as Foreman's [1914] results would suggest. The point of interest, however, is that the breakdown of glutamine cannot be due entirely to a simple hydrolysis to ammonia and glutamic acid, for the disappearance of the amino-N is greater than can be attributed to the transformation of this acid. It seems that two reactions are going on simultaneously. (1) The hydrolysis of the amide group to give ammonia and glutamic acid, the latter being transformed at once to pyrrolidonecarboxylic acid. (2) The glutamine, which is a γ-amide, is partially transformed into the anhydride, 2: 5-diketopiperazine-3: 6-dipropiondiamide, which is not so readily hydrolysed to give ammonia. This latter supposition is necessary to account for the extreme fall in the amino-N at p_H 6 and p_H 7, in which cases only small amounts of amide-N are found. The small amount of glutamine available was insufficient to permit further investigation into the chemistry of the reaction.

It was clear that the great difference in the stabilities of glutamine and asparagine at p_H 4 would enable us to estimate separately these two substances in aqueous solution. At a later stage of the research, when many data at this p_H had been accumulated, we found that urea, a possible constituent of many plant extracts, was also unstable at this p_H but very much less so at p_H 8 and the latter p_H was the one finally adopted for the estimation of glutamine in plant extracts. The small amount of glutamine available did not, however, permit us to repeat all our experiments at this p_H, so it is necessary to quote certain analyses carried out at p_H 4.

The rate of decomposition of glutamine when heated in aqueous solution at 100° (at both p_H 4 and 8) for periods of from 1 to 5 hours inclusive was next investigated; 3 hours seemed the most satisfactory period, and the data are given in Table III. It will be seen that at p_H 4 the value for amide-N is 44 % and for amino-N 5 % of the total N. The respective control values are 1 % and 86 %. The amide-N has therefore increased by 43 % and the amino-N has fallen by 81 % of the total N. Within the limits to which we shall use these figures there is no change for asparagine. If therefore a solution containing asparagine and glutamine is heated for 3 hours at p_H 4 there should be an increase of amide-N equivalent to 43 % and a decrease in amino-N equivalent to 81 % of the total glutamine-N present. The ratio decrease in amino-N/increase

in amide-N will be $\frac{81}{43} = 1{\cdot}9$. The corresponding ratio at p_H 8 will be $\frac{86-8}{35{\cdot}5-1} = 2{\cdot}3$.

We redetermined the amount of ammonia yielded by asparagine and glutamine when hydrolysed by Sachsse's [1873] method using the strength of acid and time of hydrolysis recommended by Vickery and Pucher [1931] as best for plant extracts. 30 mg. samples were boiled with $2N$ H_2SO_4 for 4 hours, and amide-N and amino-N determined *in vacuo* as before. The results are given in Table III. The amide-N in each case is about 49 % of the total N, confirming the results of previous workers.

It will be readily seen that the results given by partial hydrolysis at p_H 4 and by complete hydrolysis with $2N$ H_2SO_4 enable the amounts of asparagine and glutamine present in any given extract to be calculated in the following way.

(1) Asparagine amide-N + glutamine amide-N = increase of amide-N with $2N$ $H_2SO_4 \times \frac{50}{49}$.

(2) Glutamine amide-N = increase of amide-N at p_H 4 $\times \frac{50}{43}$.

(3) Asparagine amide-N = (1)−(2).

Analysis of a mixture of asparagine and glutamine.

Two solutions containing 120 mg. of glutamine and asparagine ($C_4H_8O_3N_2.H_2O$) respectively in 50 cc. of water were prepared. From them four other solutions were made, containing the following proportions by volume (1) 1 : 4, (2) 2 : 3, (3) 3 : 2, (4) 4 : 1. Amide-N and amino-N were determined on 6 cc. samples (*a*) under control conditions, (*b*) after heating with an equal volume of p_H 4 buffer at 100° for 3 hours, (*c*) after boiling with $2N$ H_2SO_4 for 4 hours.

The amounts of asparagine and glutamine amide-N were then calculated by means of the above equations. The agreement between the observed and actual values was excellent, as shown in Table IV.

The estimation of glutamine in plant extracts.

Before applying the above method to the estimation of asparagine and glutamine in plant extracts it was necessary to investigate certain other substances which under the appropriate conditions might give rise to amide-N or to loss of amino-N and, therefore, introduce errors.

Urea is known to be unstable in acid or alkaline solution, being converted to ammonium carbonate. Furthermore Plimmer [1925] showed that it yielded 5 % of its N as amino-N in half an hour by Van Slyke's method. Fosse [1928] and more recently Klein and Tauböck [1931] have produced evidence that this substance occurs frequently in plant extracts. We have therefore determined the stability of urea in aqueous solution when heated at 100° for three hours at various p_H values. The results are given in Tables II and III. It will be seen

Table II. *The decomposition of glutamine and other substances in aqueous solution at 100° at various* p_H *values.*

(Values given are percentages of total N.)

Substance	No. of hours hydrolysis	p_H 2		p_H 3		p_H 4		p_H 5		p_H 6		p_H 7		p_H 8		p_H 9	
		Amide-N	Amino-N	Amide-N	Amino-N	Amide-N	Amino-N	Amide-N	Amino-N	Amide-N	Amino-N	Amide-N	Amino-N	Amide-N	Amino-N	Amide-N	Amino-N
Glutamine	1	—	—	—	—	33·5	28·1	—	—	—	—	—	—	—	—	—	—
Glutamine	2*	47·7	5·5	46·3	4·6	43·0	10·0	38·8	18·8	2·8	21·5	7·8	11·8	38·8	10·1	39·2	11·0
Glutamine	3	—	—	—	—	43·7	4·1	—	—	2·6	11·0	7·3	4·1	35·5	8·3	—	—
Glutamine	4	—	—	—	—	48·2	2·0	—	—	—	—	—	—	—	—	—	—
Glutamine	5	—	—	48·6	1·9	48·3	1·0	—	—	—	—	—	—	—	—	—	—
Asparagine	2	3·8	50·2	2·8	49·0	0·4	50·0	0·3	50·8	0·8	49·3	0·7	49·3	0·6	49·1	1·4	50·2
Asparagine	5	—	—	4·8	—	1·4	48·1	—	—	—	—	—	—	—	—	—	—
Glutamic acid	3	—	74·8	—	75·8	—	84·3	—	84·1	—	78·1	—	79·0	—	72·0	—	69·1
Urea	3	—	—	—	—	34·4	5·8	20·9	12·0	1·0	19·3	2·5	14·7	5·1	4·8	6·7	4·8
i-Hydroxyglutamic acid	3	—	—	—	—	—	63·0	—	63·5	—	82·0	—	80·9	—	57·4	—	—

* These values for glutamine were obtained by boiling the solution over a free flame instead of heating on a water-bath.

Table III. *Decomposition of glutamine and other substances under various conditions.*

(Values given are percentages of total N.)

Substance	Control MgO distillation		After 3 hrs. at 100° at p_H 4		After 3 hrs. at 100° at p_H 8		Sachsse hydrolysis: 4 hrs. with $2N$ H_2SO_4		Sachsse hydrolysis: 2 hrs. with N H_2SO_4	
	Amide-N	Amino-N	Amide-N	Amino-N	Amide-N	Amino-N	Amide-N	Amino-N	Amide-N	Amino-N
Glutamine	1·1	86·3	44·0	5·0	35·5	8·5	48·5	44·0	48·9	39·1
Asparagine	0·2	50·0	0·5	49·0	1·6	48·0	48·7	47·6	48·1	49·1
Glutamic acid	—	100·0	—	84·0	—	89·8	—	—	—	—
i-Hydroxyglutamic acid	—	—	0	63·0	0	57·4	—	—	—	—
l-Leucylglutamine	—	—	1·4	27·7	0·4	32·6	—	—	32·6	33·6
i-Hydroxyaspartic acid	—	—	0	99·0	0	100·0	—	—	—	—
i-α-Hydroxyasparagine	—	—	2·0	44·0	1·9	47·5	37·3	41·0	33·3	38·9
Urea	0	10·0	34·4	5·8	5·1	4·8	37·0	7·9	22·9	5·5
Allantoin	0	1·2	2·1	0·5	3·9	2·2	22·4	5·2	3·9	4·2

Table IV. *Analysis of a mixture of asparagine and glutamine.*

Mixture number	Glutamine amide-N taken mg.	Asparagine amide-N taken mg.	Control: MgO distillation Amide-N mg.	Control: MgO distillation Amino-N mg.	After 3 hrs. at 100° at p_H 4: Amide-N mg.	Inc. in amide-N mg.	Amino-N mg.	Dec. in amino-N mg.	Ratio Dec. in amino-N / Inc. in amide-N	Sachsse hydrolysis 2N H_2SO_4 for 4 hrs. amide-N mg.	Glutamine amide-N found mg.	Asparagine amide-N found mg.
1	0·28	1·07	0·01	1·53	0·24	0·23	1·12	0·41	1·8	1·30	0·28	1·05
2	0·55	0·80	0·01	1·77	0·47	0·46	0·90	0·87	1·9	1·30	0·54	0·79
3	0·83	0·53	0·02	1·97	0·72	0·70	0·65	1·32	1·9	1·30	0·81	0·52
4	1·10	0·27	0·02	2·14	0·95	0·93	0·35	1·79	1·9	1·31	1·08	0·25

Table V. *Glutamine and asparagine contents of various plants.*

Plant	Fresh wt. g.	Aqueous extract total N g.	Control: Amide-N g.	Control: Amino-N g.	At p_H 8: Amide-N g.	At p_H 8: Amino-N g.	With N H_2SO_4: Amide-N g.	With N H_2SO_4: Amino-N g.	Glutamine amide-N g.	Asparagine amine-N g.	Dec. in amino-N / Inc. in amide-N at p_H 8	Glutamine amide-N / Dec. in amino-N with N H_2SO_4	% of total water-soluble N: Ammonia-N	% of total water-soluble N: Glutamine amide-N	% of total water-soluble N: Asparagine amide-N
Seedlings of *Ricinus communis* (grown in light)	185	0·413	0·007	0·105	0·022	0·066	0·396	0·092	0·031	0·009	2·0	1·8	1·8	7·5	2·1
Seedlings of *Ricinus communis* (etiolated)	372	0·914	0·055	0·399	0·107	0·128	0·199	0·302	0·157	0·041	2·5	1·6	6·0	17·2	4·5
Seedlings of *Brassica oleracea* (grown in light)	500	0·859	0·041	0·149	0·007	0·134	0·055	0·167	0·009	0·046	2·0	*	5·0	(1·0)*	5·4
Seedlings of *Helianthus annuus* (etiolated)	56	0·148	0·001	0·049	0·005	0·033	0·016	0·040	0·007	0·009	3·0	0·8	0·7	6·1	7·5
Roots of *Apium graveolens*	1200	0·696	0·032	0·243	0·060	0·130	0·130	0·188	0·084	0·046	1·9	1·5	4·6	12·1	6·6
Seedlings of *Vicia faba* (grown in light)	635	2·461	0·010	1·324	0·042†	1·240†	0·653	1·305	0·048	0·605	2·0	2·4	0·4	(2·0)†	24·6

* There is an increase in amino-N with N H_2SO_4, the small glutamine value is therefore rejected. † Glutamine determined at p_H 4 instead of p_H 8 (see text).

that at p_H 4 34 % of the urea is converted into ammonia and at p_H 8 only 5 %; for this reason we recommend the latter p_H when dealing with plant extracts.

Urea gives no appreciable "decrease in amino-N" at p_H 4 or 8. Consequently if it is present to any extent in plant extracts the ratio "decrease in amino-N/increase in amide-N" will be much less than 2·3.

Glutamic acid and hydroxyglutamic acid both show a decrease in amino-N at p_H 8. Since they yield no amide-N they will, if present to any extent in plant extracts, give an enhanced value for the above ratio.

Schulze [1885] showed that allantoin gave about 5 % of its N as ammonia-N on hydrolysis with N H_2SO_4 for two hours and pointed out that it was a possible source of error in the determination of the total amide-N of plant extracts. We find that it gives 4 % of ammonia-N at p_H 8 and that it gives 22 % after hydrolysis with $2N$ H_2SO_4 for 4 hours. The possible error in the determination of total amide-N in this way is considerable and we therefore recommend the milder hydrolysis employed by Schulze when dealing with plant extracts. Data are given in Table III. We have not had an opportunity of investigating the behaviour of other ureides of the type which Klein and Tauböck [1931] suggest are present in plant extracts, but they will not be less stable at p_H 8 than urea itself.

Thierfelder and von Cramm [1919] showed that *l*-leucylglutamine and other synthetic peptides containing glutamine were much more stable towards acid hydrolysis than glutamine itself. As shown in Table III we find that at p_H 8 only 0·8 % of the amide-N is liberated, so that errors due to peptides containing glutamine will be small. Hydroxyaspartic acid and α-hydroxyasparagine [Chibnall and Cannan, 1930] are also stable at p_H 8.

It will be seen that urea and ureides are the only substances we can suggest which will give an increase of amide-N at p_H 8 and glutamic and hydroxyglutamic acids the only substances which will give a decrease in amino-N at the same p_H. It is possible for these substances to be present in the same plant extract in such proportion that the ratio "decrease in amino-N/increase of amide-N" at p_H 8 is about 2·3. The error in the determination of glutamine would then be considerable.

None of these substances gives a decrease in amino-N after hydrolysis with N H_2SO_4, whereas glutamine, as shown in Table III, gives a decrease of about 50 % of the total N. We believe this large decrease in amino-N on acid hydrolysis to be a property unique to glutamine, but we have not made use of it in calculating the amount of the substance present in a plant extract because of possible errors introduced by the hydrolysis of peptides. The ratio "glutamine amide-N/decrease in amino-N with N H_2SO_4" is about 1, and if determined should effectively expose any significant error due to the combination of the four substances mentioned above.

Having thus explored the field for possible sources of error, and bearing in mind the limit of accuracy with which the amino-N of a plant extract can be

determined by Van Slyke's method, we recommend that the glutamine and asparagine content of a plant extract be determined in the following way.

(1) Glutamine amide-N + asparagine amide-N = increase in amide-N with N H_2SO_4.

(2) Glutamine amide-N = increase in amide-N at p_H 8 × 1·4.

(3) Asparagine amide-N = (1) − (2).

(4) The ratio "decrease in amino-N/increase in amide-N" at p_H 8 should be about 2·3.

(5) The ratio "glutamine amide-N/decrease in amino-N with N H_2SO_4" should be about 1. It will be raised if peptide splitting occurs during the hydrolysis.

(6) The presence of urea can be detected by an increase in the value of (2) if determined at p_H 4.

(7) The small error due to allantoin falls almost entirely on the value for asparagine amide-N.

The amounts of glutamine and asparagine in various plants.

We have determined the amide content of several plants by the above method and the results are given in Table V. The aqueous extract of the fresh plant material was prepared by the method of Chibnall [1922], except that a small mincing machine was used instead of an end-runner mill, and the final residue of cell-wall material was not extracted with boiling water. The protein in the extract was coagulated by heating as quickly as possible with steam, and removed by filtration. The clear filtrate was then rapidly cooled to 40° and concentrated to the required volume *in vacuo*. It is necessary to carry out the analyses without delay, as glutamine is unstable in aqueous solution; a test sample after standing 14 days at 15–20° gave only 64 % of the total N as amino-N. On account of the high buffering capacity of plant extracts it was found advantageous to titrate a sample with 0·1 N sodium hydroxide either electrometrically to p_H 8 or to a faint pink with phenolphthalein.

Ricinus communis (*castor-oil plant*). The 21-day etiolated seedlings contained a high proportion of amide-N, the major part of which was due to glutamine. Schulze [1897] obtained considerable amounts of glutamine, but was unable to isolate any asparagine.

As was expected, the seedlings grown in the light contained less total amide-N, but the major part was again due to glutamine. It is interesting to note that Green [1890] isolated a small amount of asparagine from young embryos germinating in the light.

Brassica oleracea *var.* bullata (*Brussels sprout*). The young seedlings grown in light did not contain much amide-N, and the major part of this was due to asparagine. Although the ratio "decrease in amino-N/increase in amide-N" at p_H 8 is 2, the small value (1·0 %) for glutamine amide-N is rejected because there is an increase in amino-N with N H_2SO_4 instead of the decrease which

should prove the presence of glutamine. As was pointed out in the earlier discussion, if the glutamine content is small the "decrease in amino-N with N H_2SO_4" may be masked by peptide splitting. It is probable that this has occurred in the present case, because Schulze [1897] was able to isolate asparagine, and with great difficulty a very small amount of glutamine from *B. oleracea* var. *gongylodes*.

Helianthus annuus (*sunflower*). Schulze's pupil Frankfurt [1893] was able to isolate asparagine and glutamine from etiolated seedlings. Our results confirm the presence of both amides.

Apium graveolens (*celery*). Schulze [1897] isolated asparagine and glutamine from the roots. Again our results confirm the presence of both amides.

Vicia Faba (*broad bean*). Schulze, in a series of papers, showed that seedlings, especially etiolated seedlings, of the Leguminosae accumulate large quantities of asparagine, but he was never able to isolate glutamine. Our results for the seedlings of the broad bean grown in daylight show the presence of large amounts of asparagine. The increase in amide-N at p_H 4 (this experiment was made before the procedure was changed to p_H 8) is very small and is of the same order as that which would be given by the asparagine itself. The observed value for glutamine amide-N is therefore considered to be within the experimental error of the method and is rejected.

It will be seen from the above results that the micro-method described in this paper, which requires only 1–3 g. of fresh plant material, estimates to within an error of about 1 in 10 the amount of glutamine in the aqueous extract. Furthermore instability of this substance in aqueous solution, which has been clearly demonstrated, offers a ready explanation of the low yields, compared with that suggested by Sachsse-hydrolysis, of crystalline glutamine which Schulze always obtained from plant extracts.

Summary.

Attention is called to the fact, previously discovered by Schulze and Thierfelder, that glutamine reacts abnormally with nitrous acid. It gives 92 % of its total N as amino-N in the Van Slyke apparatus.

The stability of glutamine in solution at 100° at various p_H values has been investigated by determining both amide-N and amino-N.

After heating at 100° at p_H 8 for 3 hours glutamine gives 35 % of the total N as amide-N, while the amino-N falls to 8·5 %. Asparagine is stable under these conditions.

This difference in behaviour has been made the basis of a method for the separate determination of asparagine and glutamine in plant extracts.

The method has been applied with success to a mixture of asparagine and glutamine, and also to 6 plant extracts, the amide contents of which were known from the previous work of Schulze.

In conclusion we should like to thank Prof. Winterstein for the gift of 2 g. of glutamine, Prof. Knoop for a sample of *l*-leucylglutamine synthesised by the late Prof. Thierfelder and Prof. Harington for a sample of synthetic *i*-hydroxyglutamic acid.

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XVI. GROWTH OF *ASPERGILLUS VERSICOLOR* ON HIGHER PARAFFINS.

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Introduction.

In connection with some investigations which are being made on the metabolism of paraffins in the plant it became of interest to find out the chemical changes which these substances undergo when used as the sole source of carbon for the growth of moulds.

It was shown by Tausson [1925] that a mould resembling *Aspergillus flavus* could utilise paraffin wax for this purpose, and by Tausz and Peter [1919] that certain bacteria isolated from the soil could also utilise synthetic paraffins. In the present research we have been fortunate enough to isolate another mould which will grow on paraffin wax. It is a member of the *A. versicolor* group and we have investigated its action on certain synthetic long-chain paraffins, with the hope that some information would thereby be gained as to the manner in which the organism attacks a long carbon chain of this type, which has no polar group.

By analogy with the action of moulds on fatty acids we should expect the chain to be broken at some particular carbon atom by oxidation. Stokoe [1928] showed that *Penicillium palitans* growing on a gelatin medium containing deodorised coco-nut oil oxidised the fatty acids at the β-carbon atom. Normally the β-keto-acids thus formed broke down to give the fatty acid containing two carbon atoms less and acetic acid, but under certain conditions, as for instance when respiration of the mycelium was impeded, the keto-acid lost carbon dioxide with the formation of a methyl ketone. Similar evidence was obtained by Acklin [1929]. Walker *et al.* [1928], who grew *A. niger* on calcium butyrate, suggested that the initial product of the oxidation was the β-hydroxy-acid. Dakin [1908, 1, 2; 1909], by acting on the ammonium salts of

fatty acids *in vitro* with hydrogen peroxide, showed that oxidation took place at the β-carbon atom, and that small amounts of the corresponding methyl ketones were also formed. He did not consider, however, that a definite answer could be given to the question whether an unsaturated acid, β-hydroxy-acid or β-keto-acid, was the first product of oxidation, and suggested that in the animal body all three would be in equilibrium with each other and readily interconvertible. These experiments have been repeated more recently by Raper and Clutterbuck [1925], who showed that in addition oxidation also took place at the γ- and δ-carbon atoms, with the formation of the corresponding keto-acids.

A suggestion as to the possible course of biological oxidation of the long-chain paraffins can be obtained from the intensive study of Francis *et al.* [1922, 1, 2] on the air oxidation of paraffins. They examined the products obtained by treatment of paraffin wax with air and oxygen at 100° in the presence of small amounts of turpentine to hasten the onset of oxidation. Although the products obtained were very complex mixtures the presence of secondary alcohols and ketones was established; it was probable also that dihydric alcohols, hydroxyketones and diketones were present. Primary alcohols were not detected. The secondary alcohols and ketones obtained probably had the same number of carbon atoms in the molecule as the paraffins originally present in the wax from which they were derived. The ketones were considered to be derived from the secondary alcohols, which were the primary products of oxidation, and an X-ray examination of the ketones showed that the carbon chain was attacked by oxygen at the third or fourth atom from the end. In addition to these non-acidic substances, complex mixtures of acidic substances were obtained. These had in all cases shorter carbon chains than the paraffins present in the original paraffin wax. Later, Francis and Wood [1927] examined the products obtained by oxidation of synthetic *n*-triacontane, $C_{30}H_{62}$, under similar conditions, and were able to establish the presence of 4-triacontanone, $CH_3.(CH_2)_{25}.CO.(CH_2)_2.CH_3$, an acid containing 26 carbon atoms and succinic acid. Evidence was also obtained that the primary oxidation product was a secondary alcohol. Since the amounts of these substances obtained were very small it was thought probable that the monohydroxy-derivative first formed was largely oxidised further to polyhydroxy-derivatives, which on subsequent oxidation gave polyketones, and then acids and hydroxy-acids with shorter carbon chains.

Tausson [1925, 1928], as the result of his experiments on the growth of *A. flavus* on paraffin wax, concluded that one of the first steps in the oxidation of the paraffins by the mould was the production of the fatty acid esters of the higher aliphatic alcohols. He considered that this view was supported by the fact that the mould would grow on such esters under the same conditions as on paraffin wax. Since, however, it is hardly conceivable that the mould could produce an ester from paraffin wax direct, without the preliminary production of its constituent acid and alcohol, it does not seem possible for esters to be

directly concerned with the metabolism of paraffin hydrocarbons. Further criticism of Tausson's work is given later.

In the present work attempts were made to grow a number of moulds on pure synthetic paraffins. A strain of *A. versicolor* was eventually obtained which would grow on these substances as a sole source of carbon. The mould grew equally well on odd- or even-number paraffins, but there was an upper limit to the length of the chain which it would attack; for only with great difficulty could growth be obtained on *n*-tetratriacontane $C_{34}H_{70}$, and it was never found possible to obtain growth on *n*-pentatriacontane $C_{35}H_{72}$.

An investigation has been made into the products of metabolism when the carbon source has been *n*-heptacosane $C_{27}H_{56}$. Only extremely small amounts of metabolic products were obtained, and these were apparently for the most part complex mixtures. These results suggested that the carbon chain had been attacked at numerous points, with the ultimate production of carbon dioxide and, perhaps, water-soluble substances of quite low molecular weight. In this connection it is noteworthy that Raistrick and his co-workers [1931], in the course of an investigation of the metabolic products of a very large number of mould species grown on a glucose medium, found that by far the greater number produced practically nothing but carbon dioxide as a metabolic product.

It is possible that by restricting the oxygen supply the paraffin metabolism of the mould could be diverted from its normal course, so as to lead to the accumulation of either direct intermediary products or others derived from them; just as, for instance, Stokoe [1928] found that methyl ketones were produced from fatty acids when the respiration of the mould was hindered by the presence of excess of fat. Unfortunately the normal growth of *A. versicolor* on paraffins is very slow, and such an experiment might easily lead to so little growth that intermediary products would be produced in quantity insufficient for chemical identification. It seemed to us that information might be more easily obtained by growing the mould on products which might themselves be intermediaries in the metabolism of paraffins.

The following substances were chosen for this purpose:

2-pentadecanone $CH_3.(CH_2)_{12}.CO.CH_3$,
12-tricosanol $CH_3.(CH_2)_{10}.CHOH.(CH_2)_{10}.CH_3$,
14-heptacosanone $CH_3.(CH_2)_{12}.CO.(CH_2)_{12}.CH_3$,
14-heptacosanol $CH_3.(CH_2)_{12}.CHOH.(CH_2)_{12}.CH_3$,
15-nonacosanol $CH_3.(CH_2)_{13}.CHOH.(CH_2)_{13}.CH_3$,
d-10-nonacosanol $CH_3.(CH_2)_8.CHOH.(CH_2)_{18}.CH_3$,
16-hentriacontanone $CH_3.(CH_2)_{14}.CO.(CH_2)_{14}.CH_3$,
16-hentricontanol $CH_3.(CH_2)_{14}.CHOH.(CH_2)_{14}.CH_3$,
and myricyl alcohol, M.P. 88°, from carnauba wax.

Normal growth occurred with the primary alcohol and the ketones, but no growth at all with the secondary alcohols. These results suggest that the biological oxidation of paraffins takes place in the first instance by the direct

introduction of a keto-group, perhaps at several points in the chain simultaneously. The oxidation then causes disruption of the paraffin chain with the formation of shorter fatty acids, which are then further metabolised through keto-acids, as discussed previously. It will be seen that the scheme of metabolism excludes the initial formation of secondary or polyhydric alcohols, and in this respect only does it differ from the air oxidation of paraffins observed by Francis. On general chemical grounds it is, of course, highly improbable that the paraffin chain could be oxidised at the terminal carbon atom with the formation of a primary alcohol or *n*-fatty acid containing the same number of carbon atoms.

We reject Tausson's [1925] conclusion—that one of the first steps in the oxidation is the production of fatty acid esters of the higher aliphatic alcohols—for the following reasons.

(1) In his experiments the amount of intermediary products formed was very small. From one culture after 12 weeks' growth on 3·65 g. of paraffin there were obtained 1·24 g. of mycelium and 1·29 g. of residual paraffin. The latter was extracted at 70° with 70 % alcohol, when an intense orange-yellow solution was obtained. This contained 0·234 g. of products which were stated to be non-acidic. Another sample of residual paraffin weighing 1·03 g. was saponified directly with 1 % aqueous potassium hydroxide, when 0·0178 g. of an acidic product was obtained. Without further characterisation this was assumed to be fatty acid derived from esters of higher alcohols. Such esters are not readily saponified by this mild hydrolysis and, as Tausson admits that much decomposition took place with evolution of gaseous products having the smell of terpineol, we suggest that this small amount of acid was perhaps derived from the pigments present, for in our own experiments these were acidic.

(2) No steps were taken to ensure that the paraffin wax used was free from traces of other substances, or to carry out blank estimations on the paraffin used. We have found that commercial paraffin wax blackens considerably when treated with concentrated H_2SO_4 at 120°, and therefore contains at least a small amount of material that is not true paraffin.

(3) As the dried mixture of mycelium and residual paraffin was extracted with ether, the extract possibly contained any ether-soluble material present in the mycelium, and hence any fats found may not have been intermediate products but essential constituents of the mycelium.

Experimental.

Preparation of paraffins, ketones and alcohols.

These were synthesised by the methods described in a previous paper [Piper *et al.*, 1931]. *n*-Heptacosane was the paraffin chosen for the chief experiments on the metabolic products because ample supplies of myristic acid, from which it is prepared, were available. The paraffins, ketones and alcohols were prepared for the cultures by slow crystallisation from hot benzene-alcohol

(1 : 9), the thin flat crystalline leaflets being filtered off by very gentle suction, so as to prevent them packing down, and air-dried. By this means material exposing a considerable surface to the action of the mould was obtained. The paraffin wax used in the later experiments was commercial wax melting at about 50° which had been treated with concentrated H_2SO_4 at 120° until no further blackening took place and then recrystallised from benzene-alcohol to obtain it in a finely divided state. Its melting-point after treatment was 53–55°.

Isolation of a mould which will grow on paraffin wax.

In Tausson's experiments cultures were prepared by adding sterile scrapings of paraffin wax (M.P. 78°) to a flask containing sterile mineral salt solution and then inoculating. The mould was grown at 23–25°. Development of mycelium was first noticeable after 10 days, and, as growth proceeded, yellow and finally red pigment was produced. Change of p_H of the medium was found to have little effect on the rate of growth, acid media tending to become more alkaline as growth proceeded. Nitrates and ammonium salts served equally well as sources of nitrogen.

The mould used by Tausson was stated by him to be similar to *Aspergillus flavus*, and as a preliminary experiment it was decided to attempt to grow this and a number of related moulds on paraffin wax. Through the kindness of Prof. Raistrick we were enabled to obtain cultures of the following moulds from Messrs Nobel's Ltd. of Ardeer: *A. flavus*, *A. parasiticus*, *A. oryzae*, *A. effusus*, *A. tamari* and an unnamed *Aspergillus*, numbered Ac 62.

Cultures of these were prepared by adding 2 g. of paraffin wax (M.P. 57°), cut into shavings by means of a microtome, to 50 cc. of mineral salt solution contained in each of a number of litre flasks. The mineral nutrient solution had the following composition: $Ca(NO_3)_2$ 0·1 g., KNO_3 0·025 g., KH_2PO_4 0·025 g., $MgSO_4$, $7H_2O$ 0·025 g., $Fe_2(SO_4)_3$ 0·0025 g., H_2O 100 cc. Each flask was then heavily inoculated with the appropriate mould. The method of inoculation was to take a vigorously growing culture on a beerwort or glucose agar slope, add about 60 cc. of mineral nutrient solution, and scrape up as much of the mould as possible with a platinum wire without disturbing the agar. The suspension of mycelium and spores thus obtained was poured into the prepared flask, and distributed by gentle shaking. It was not thought necessary to carry out these experiments under strictly sterile conditions. The flasks were then incubated at about 28°.

Very slight growth appeared to take place in all cases, although *A. flavus*, *A. effusus* and *A. tamari* were the most promising. Further experiments were therefore made with these three moulds, using *n*-heptacosane as source of carbon instead of paraffin wax. Nine flasks were prepared, each containing 1 g. of *n*-heptacosane and 25 cc. of mineral nutrient solution; and 3 were inoculated with *A. effusus*, 3 with *A. flavus* and 3 with *A. tamari*. The paraffin was prepared in fine leaflets, as stated above, and was readily distributed over the whole surface of the liquid in the culture flask. After 3 weeks there was

little evidence of growth, except in the case of one flask inoculated with *A. tamari*, on the side of which had appeared a small green patch surrounded by some red pigment. The remaining flasks were therefore re-inoculated with the appropriate moulds, using for inoculation vigorously growing 6-day subcultures on beerwort agar. After 2 days the flasks re-inoculated with *A. tamari* showed signs of growth and continued to grow; none however of the other cultures showed any growth.

Three weeks after the second inoculation the amount of paraffin remaining in one of the *A. tamari* flasks was determined in the following way. The culture was washed on to a Büchner funnel, and the mixture of paraffin and mycelium then removed from the filter-paper and treated with successive portions of warm light petroleum (B.P. 40–60°) to remove all the paraffin. This solvent will not remove any fatty material from the fresh mycelium, whereas ether, the solvent employed by Tausson, might readily do so if employed in quantity sufficient to bring about partial dehydration of the mycelium. The weight of mycelium, dried at 100°, was 0·046 g. and of the recovered paraffin 0·92 g. This did not blacken when treated with concentrated H_2SO_4 at 120°, and was identical in all respects with the original paraffin.

This, and many similar results were so discouraging as to cause us to abandon the experiments with moulds of known history, and, as a last resort, to carry out experiments with the unknown green mould which had, as has been previously mentioned, appeared on the side of one of the culture flasks.

A subculture was made from this patch of mould direct into another flask containing 1 g. of *n*-heptacosane and mineral nutrient solution. After 6 days patches of red pigment appeared on the paraffin adhering to the walls of the culture flask, and later, green areas of mould. Growth also took place on the paraffin floating on the surface of the mineral nutrient solution, but was not noticeable so early as that on the walls, because red pigment was not at first produced. After some weeks, however, the mineral nutrient solution became pink in colour.

Glucose agar slopes were inoculated with material from this culture and incubated at 28°, and by this means subcultures were obtained which appeared to be fairly pure. From these Prof. Raistrick kindly prepared pure cultures for us by plating out on glucose agar. The course of development of the mould on this medium was as follows. After 2 days, white patches of mycelium appeared which turned yellow or orange as growth proceeded, the centres of the patches then turning green owing to production of spores. After about 2 weeks the agar, which had at first remained colourless, became dark red.

Through the kindness of Prof. Raistrick, a culture of the mould was sent to Prof. Thom, of Washington, D.C., U.S.A., who reported that it appeared to be a member of the *A. versicolor* group. This organism, which is best described at the present time as a strain of *A. versicolor* (Buillemin) Tiraboschi, appeared to be a suitable one for further experiments, and the products formed by its action on *n*-heptacosane were therefore examined.

Examination of products formed by the action of a strain of A. versicolor *on* n*-heptacosane.*

After 8 weeks, the first subculture of *A. versicolor* on *n*-heptacosane was examined for products of metabolism. The mineral nutrient solution was removed by filtration, and the residue was extracted several times with light petroleum (B.P. 40–60°). The extract was evaporated to dryness; weight 0·849 g. The mycelium was dried for some days *in vacuo* over $CaCl_2$; weight 0·068 g.

The product recovered was yellow in colour, and a sample blackened somewhat when heated with concentrated H_2SO_4 to 120°. It was redissolved in ether, and the solution extracted three times with a dilute solution of sodium hydroxide. The washings were bright pink. The ethereal solution was now much paler yellow than before, but further washing removed no more pigment. After removal of the ether the product melted at 58·8–59·1°, and obviously consisted largely of unchanged paraffin. It was therefore recrystallised from about 50 cc. of benzene-alcohol (1 : 2) yielding a colourless material similar in all respects to the original paraffin. The mother-liquor was evaporated to dryness and the residue, which weighed only 0·05 g., melted indistinctly and was completely molten at 58·5°, the melt being cloudy. It still probably contained much unchanged paraffin, and, as the total amount was small, it was not examined further until it had been combined with similar products from later cultures.

An experiment was carried out to determine whether change of p_H of the mineral nutrient solution towards greater alkalinity had any effect on the rate of decomposition of the paraffin by the mould. Two flasks, each containing 1 g. of *n*-heptacosane and 100 cc. of mineral nutrient solution, were inoculated with *A. versicolor* grown on glucose agar, and to one, excess of powdered $CaCO_3$ was added. The flasks were incubated at 28° for 5 weeks, and the amount of paraffin remaining was determined as previously described. With $CaCO_3$, 0·64 g.; without $CaCO_3$, 0·62 g.: the two products were similar.

Thus change of initial p_H from 5·8 to 7·9 had no effect on the rate of oxidation of the paraffin, and in all later experiments the original nutrient solution of p_H 5·8 was used. It was found that cultures on this nutrient solution slowly became more alkaline, the reaction at the end of the experiments being about p_H 8.

In another experiment, a culture on *n*-heptacosane which had begun to develop at 28° was removed from the incubator and allowed to develop at room temperature. Growth was visibly slower than in cultures kept at 28° and the latter temperature was therefore used in subsequent experiments.

Examination of a large culture. Eighteen cultures, each on 1 g. of *n*-heptacosane, were prepared in the same way as before and allowed to develop for 5–6 weeks. They were then filtered off and extracted with light petroleum as previously described. The weight of residual paraffin was 10·47 g. The mycelium was dried over $CaCl_2$; it weighed 3·416 g. Thus more than 4 g. of

n-heptacosane had been oxidised to CO_2 or water-soluble substances of low molecular weight.

The light petroleum extract was dark yellow. It was dissolved in ether and the ethereal solution washed with a 5 % solution of sodium hydroxide. The alkaline washings were deep purple. The washed ether solution, which was now pale yellow, was evaporated to dryness and the residue dissolved in 1300 cc. of a boiling mixture of benzene and alcohol (1 : 2), and the solution allowed to cool. The major part of the material crystallised out, and was shown by its melting-point and behaviour towards concentrated H_2SO_4 at 120° to be unchanged *n*-heptacosane. The mother-liquor, on evaporation to dryness, yielded a product weighing 1·7 g. which was not homogeneous, and contained brown gummy material. This was dissolved in 150 cc. of hot benzene-alcohol, and the solution allowed to cool. The product which crystallised out was again shown to be pure *n*-heptacosane. The mother-liquor on evaporation to dryness yielded 0·282 g. of material still containing much *n*-heptacosane. The fractionation was continued using first 30 cc. and finally 5 cc. of benzene-alcohol; 0·121 g. and 0·020 g. of practically pure *n*-heptacosane being obtained. The final mother-liquor yielded 0·14 g. of brown gummy material which could not be fractionated, and, owing to the small amount available, it was not examined further.

The purple alkaline washings from the above were acidified with HCl, when the solution turned orange. It was extracted with ether, and the ethereal solution evaporated to dryness. Rather less than 0·1 g. of red non-crystalline material was obtained.

The dried mycelium. This was powdered in a mortar and extracted with ether in a Soxhlet apparatus. The orange-coloured extract, after washing with a dilute solution of sodium hydroxide, yielded 0·318 g. of pale yellow material which was solid and largely crystalline. The washings were intense purple in colour, and, on acidification and extraction with ether, gave 0·16 g. of red acidic products, from which it was not found possible to obtain crystalline material.

From the yellow crystalline substance there was obtained, by recrystallisation from alcohol, 0·021 g. of material consisting of yellow needles, together with some unchanged *n*-heptacosane. This material was insoluble in ether and melted at 213·5–214·5°. It contained no nitrogen. (Found: C, 64·3; H, 4·14 %. Mol. wt. (depression of M.P. of camphor) 368. $C_{19}H_{14}O_7$ (Mol. wt. 354) requires C, 64·4; H, 3·95 %.) It is interesting to compare this rather unusual empirical formula with those of citromycetin ($C_{14}H_{10}O_7$; $2H_2O$) and citrinin ($C_{13}H_{14}O_5$), two yellow crystalline compounds described by Raistrick *et al.* [1931] as having been formed by the fermentation of glucose by moulds.

A second batch of 16 cultures was examined after growing for 3–4 months and yielded similar results. The main paraffin fraction, however, was further divided into a number of fractions by crystallisation from a mixture of acetone and light petroleum (1 : 2) with a view to detecting any substance which had a greater or less solubility than *n*-heptacosane in this solvent. No such substance was found.

Experiments on the growth of A. versicolor *on other paraffins.*

Other paraffins available were *n*-tricosane, *n*-nonacosane, *n*-triacontane, *n*-tetratriacontane and *n*-pentatriacontane. Two cultures of each were made. In the case of *n*-nonacosane both were inoculated from glucose agar cultures, and in all other cases one was inoculated from a glucose agar culture, and one from a vigorously growing beerwort agar culture. The results of these experiments are given in Table I.

Table I. *Showing the growth of a strain of* Aspergillus versicolor *on a series of long-chain paraffins.*

Paraffin	Culture from which inoculated	Time in days before growth was observed	Description of growth
$C_{23}H_{48}$	Glucose	8	Good growth with very little production of pigment
$C_{23}H_{48}$	Beerwort	6	
$C_{29}H_{60}$	Glucose	7	Growth and pigment production similar to that using *n*-heptacosane
$C_{29}H_{60}$	Glucose	7	
$C_{30}H_{62}$	Glucose	8	Growth and pigment production similar to that using *n*-heptacosane
$C_{30}H_{62}$	Beerwort	6	
$C_{34}H_{70}$	Glucose	—	No growth obtained
$C_{34}H_{70}$	Beerwort	12	Very slow growth
$C_{35}H_{72}$	Glucose	—	No growth, even after re-inoculation
$C_{35}H_{72}$	Beerwort	—	
Paraffin wax	Glucose	8	Good growth with very little production of pigment
Paraffin wax	Glucose	8	

It will be seen that mould growth has taken place on both odd- and even-number paraffins but that no growth has occurred on paraffins higher in the series than *n*-tetratriacontane.

Experiments on the growth of A. versicolor *on ketones and alcohols.*

These were carried out in the usual way with vigorously growing glucose agar cultures. A control experiment with *n*-heptacosane was made at the same time. Less pigment is produced in the case of the ketones, which makes the observation of initial growth more difficult; this is probably the reason for the longer latent period recorded for some of these substances in Table II. The results have been fully discussed in the Introduction.

Table II. *Showing the growth of a strain of* Aspergillus versicolor *on a series of ketones and alcohols.*

Substrate	Time in days before growth was observed	Description of growth
2-Pentadecanone	9	Normal growth, very little pigment observed
12-Tricosanol	—	No growth
n-Heptacosane (control)	14	Normal growth
14-Heptacosanone	21	Normal growth, very little red pigment formed
14-Heptacosanol	—	No growth
15-Nonacosanol	—	No growth
d-10-Nonacosanol	—	No growth
16-Hentriacontanone	21	Normal growth, very little red pigment formed
16-Hentriacontanol	—	No growth
Myricyl alcohol from carnauba wax, M.P. 88°	21	Growth not so good as on paraffins and ketones

SUMMARY.

A new mould has been found which will grow on synthetic higher paraffins as sole source of carbon. It is best described at the present time as a strain of *Aspergillus versicolor* (Buillemin) Tiraboschi.

It will grow on both odd- and even-number paraffins, but not on those with a longer chain than $C_{34}H_{70}$.

Experiments have been made to find out the way in which the paraffin chain is attacked by the mould, but a large scale culture on $C_{27}H_{54}$ did not yield any products of metabolism except carbon dioxide and mould mycelium. Indirect evidence has, however, been obtained from experiments with higher ketones on which the mould grew vigorously, and with secondary alcohols on which no growth was obtained at all. It appears that the primary product of oxidation of a paraffin is a ketone or polyketone, and that further oxidation results in the production of shorter fatty acids which are then metabolised in the usual way.

In conclusion we should like to express our cordial thanks to Prof. Raistrick for the gift of many species of *Aspergillus* and for advice and assistance in preparing the cultures.

We should also like to thank Prof. Garner for samples of *n*-triacontane and *n*-tetratriacontane, and Prof. Thom for the identification of the mould.

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XVII. THE BROMINE REACTION OF PREGNANCY URINE.

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Some two years ago Voge [1929] suggested that Knoop's test for histidine, when applied to urine, offered a simple means of testing for pregnancy. With the efficacy of this test we are not concerned in this communication. Reports upon it have been supplied by Burt-White [1930] and Voge [1930]. We became interested in this reaction at the instigation of Dr R. Greene, who has been applying it clinically and will be publishing his results shortly. In an attempt to render the reaction more decisive we were led to inquire whether the substance in urine giving a positive Knoop test was, as is always assumed, histidine. As a result, we have found that the substance concerned is histidine and we have no evidence that any other substance is involved in the reaction.

Knoop's test consists in adding bromine water to the solution under test to the point of maximum absorption, avoiding excess. On heating, a port wine-red colour develops which fades slowly on standing, a black particulate precipitate being deposited. The reaction works best in faintly acid solution; the colour is modified but not inhibited by the presence of strong acids. Hunter [1922, 1] has rendered the reaction more sensitive.

Isolation of histidine.

After some preliminary experiments a Kossel silver fractionation was adopted, with slight modifications, the bulk of the substance being found in the histidine fraction and a little in the arginine fraction. The process is very similar to that described by Hunter [1922, 2].

The urine used was always a mixed sample and was frequently contaminated with debris. It was early found desirable to give it a preliminary clearing with baryta, which (*a*) rendered filtration more rapid, (*b*) removed phosphates which would otherwise use up silver and (*c*) removed a certain amount of pigment. Hot saturated baryta was added to the urine to the point of maximum precipitation. The precipitate was allowed to settle and the mixture filtered on a Büchner funnel, the precipitate being added last. Strong nitric acid was then added to the filtrate until the reaction was just acid to Congo red. Silver nitrate was added in the form of a 60 % solution until no further precipitation took place. The precipitate (mainly silver chloride) was allowed to settle and

was then filtered off through a fluted filter-paper, filtering the supernatant fluid first. More silver nitrate solution was added to the filtrate until it gave the brown spot test with baryta. Strong NaOH solution was then added to bring the solution to p_H 7·4. The precipitate was allowed to settle; the supernatant liquid was decanted and the silver precipitate washed twice with distilled water, by decantation. It was then suspended in water, made acid, and the silver fractionation repeated. The silver precipitate so obtained was again suspended in water, made acid to Congo red with sulphuric acid, and treated with hydrogen sulphide. The silver sulphide was filtered off and the filtrate aerated and finally just boiled to expel the hydrogen sulphide. Whilst still hot an appropriate amount (about 1 g. per 100 cc. of liquid) of charcoal was added, with stirring. The mixture was filtered, and the sulphuric acid was removed from the filtrate quantitatively with just sufficient baryta. The clear colourless filtrate was then concentrated by distillation *in vacuo* to about 1/30 of its volume. At this stage it was found desirable to treat the concentrate with picric acid in order to remove creatinine, which otherwise followed the fractionation through to the end. Difficulties were encountered in trying to remove the excess of picric acid by ether extraction, and in the end it was necessary to resort to a further silver precipitation, the silver salt being subjected to a very thorough washing by decantation in order to remove picric acid. The silver salt was decomposed in the usual way, the filtrate freed from sulphuric acid and baryta and concentrated almost to dryness by distillation *in vacuo*. The syrupy residue was transferred to a beaker, the distilling flask being rinsed with a small amount of 50 % HCl and the rinsings were added to the main fraction. Boiling 97 % alcohol was then added to the syrup and the mixture kept boiling for ten minutes. If at all coloured, a small amount of charcoal was added at this stage. The solution was filtered hot. When cool, ether was added to the filtrate until permanent cloudiness was obtained. If now left at 0° the product usually separated as a syrup. Such a syrup was dried and redissolved in hot 90 % alcohol containing 5 % HCl, from which the crystalline product separated on cooling. This was the general scheme for the separation of the substance giving the Knoop reaction. It was frequently necessary at the last stage to dissolve in alcohol and to precipitate several times with ether, to vary the strength of the alcohol, or to extract the syrup with absolute alcohol, in order to obtain a crystalline product. An attempt to obtain a dihydrochloride by the method used by Vickery and Leavenworth [1928] for histidine was only partially successful, a mixture of mono- and di-hydrochlorides being apparently obtained.

The yield of crystalline material was very small, 0·2 g. being obtained from some ten litres of urine. There was undoubtedly much wastage, the syrupy non-crystallisable residue rejected in the end stages being very rich in material giving a positive Knoop reaction. It is probably safe to assume that the initial concentration was of the order 0·7 to 1·0 g. in the ten litres.

Identification.

There could be little doubt from its general properties and its occurrence in the histidine fraction of the silver precipitation that the substance isolated was histidine. The crystalline appearance of the hydrochloride (thick rhombic prisms) afforded confirmation of this. The melting point was 251° (the melting point of histidine monohydrochloride is usually given as 251–252°). In addition it showed that curious effervescence at 165–170° without melting, which is characteristic of histidine hydrochloride. A mixed melting-point with a purchased specimen of histidine hydrochloride showed no depression. There was, however, always the possibility of the substance being methylhistidine; the melting-point of the hydrochloride of this compound is not known to us and the melting-point of the base itself is given [Linneweh, Keil and Hoppe-Seyler, 1929] as 248–252°; the melting-point of histidine itself is 253°.

Analyses were therefore carried out. The results were:

N: 20·6, 19·55 (selves), 18·4 (Schoeller) %.
N: calculated, 20·05 %.
Cl: found, 17·36 (selves), 16·67 (Schoeller) %.
Cl: calculated, 16·94 %.

The slightly low result for nitrogen by Schoeller is discrepant. Our nitrogen analyses were done by the micro-Kjeldahl method and with a purchased specimen of histidine we obtained 19·17 % N. We conclude therefore that the substance isolated is histidine.

Specificity of Knoop's reaction.

In the course of this work we have carried out a large number of Knoop tests and we have applied this reaction to many substances other than histidine. In particular we have tested many compounds closely related structurally to histidine (*i.e.* glyoxaline derivatives) and we have in other cases culled from the literature references to the application of this test. No other substance in our experience gives a Knoop reaction similar to that of histidine and the only instance quoted in the literature is that of methylhistidine [Linneweh *et al.* 1929]. Thus α-hydroxy-β-iminazolylpropionic acid in our hands gives no Knoop test; nor, on the other hand, does iminazolylacetic acid [Stewart, 1923]. Fargher and Pyman's [1921] methylhistidine however does not give a positive Knoop test; but in this case the methyl group is attached to the amino-group, whereas in Linneweh, Keil and Hoppe-Seyler's methylhistidine the methyl group is attached to the ring. A few substances have been met which give with bromine water a reaction of a different type; *e.g.* adrenaline gives a rose-red colour, but quickly and without the application of heat. Tyramine likewise gives a somewhat similar reaction, but this may be differentiated by the initial formation of a cloudy solution. Knoop [1908] himself tested several glyoxaline derivatives and concluded that the only substance

other than histidine giving a positive test was histamine; but he adds "...doch ist das Verhalten hier etwas abweichend." That is precisely our experience, the reaction with histamine being quite different, because (*a*) the colour obtained is violet and (*b*) it can be readily extracted with amyl alcohol (with histidine, this is not the case). Apart, therefore, from methylhistidine, we are convinced that with due care and experience Knoop's test is specific for histidine.

SUMMARY.

1. The substance in pregnancy urine giving a positive reaction with bromine water (Knoop's test) has been isolated and identified as histidine.

2. Knoop's test is considered to be specific for histidine and *N*-methylhistidine.

We are indebted to Prof. Peters for his advice and encouragement.

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XVIII. A TECHNIQUE FOR STUDYING THE METABOLISM OF RATS.

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(*Received January 1st, 1932.*)

It is very important that the following conditions should be observed if accurate results are to be obtained in metabolism experiments. First, during the whole experimental period the daily intake of food must be the same, secondly, the food given must be of constant chemical composition, and, thirdly, all the excretions must be accurately collected.

The technique used for studying the metabolism of rabbits and dogs has been described elsewhere [Korenchevsky, 1925, 1, 2]. In this paper are summarised the technical requirements necessary for obtaining accurate results with the rat.

It was necessary to overcome the following difficulties of technique.

1. Rats are small animals, therefore their metabolism is readily influenced by changes in the temperature of their environment. Our experience has shown that a difference of 2° in room temperature affects the metabolism of the rat considerably.

2. Rats are easily excited by sounds and movements in their environment. Differing degrees of physical activity during different periods of the experiment influence particularly the gaseous metabolism.

3. Rats usually scatter their food over the floor of the cage. Therefore in the metabolism cage food is dropped into the urine and faeces containers, making accurate investigation of the metabolism impossible because (*a*) the ration will not have been entirely eaten, (*b*) the food mixed with urine or faeces will render the analysis of excreta inexact.

4. Rats do not usually consume their food in a limited time, but when feeding naturally eat at intervals during the whole 24 hours. The time of feeding, however, influences the metabolic rate.

5. The exact separation of the urine and faeces of the different periods of the experiment is difficult since it is not possible to use the catheter or to stain the food with colouring matter and through the food the faeces of alternate experimental periods.

To overcome the first two difficulties mentioned a special room is set aside for metabolism experiments only. A room in the middle of the building and having no windows is used. This room is kept at a constant temperature of

25° by means of a thermoregulator connected with an electric radiator. The ventilation is reduced to such an extent that, whilst the minimal requirements of ventilation are met, the temperature is kept constant even when the outside temperature changes very considerably. The room is entered only at special times each day and only for the purposes of experiment, so that the rat is disturbed as little as possible. The lack of daylight is repaired by exposing the rats twice a week to the ultra-violet rays of a mercury vapour lamp.

To overcome the difficulties mentioned in §§ 3 and 4, only those rats which can be trained to consume their food in about 5 hours from the time the food is given are chosen for metabolism experiments. During the whole period of the experiment the food is given daily at the same time, in the same amount and of the same composition. In order to prevent the rats from dropping their food in the cage they are given a semi-liquid paste. By this simple device the rats are able to lick the paste from the dish but are not able to take it in their paws and scatter it in the cage. The paste used lately is of the following composition: caseinogen 18, starch 49, butter 13, salt mixture 5, dried yeast 10, sugar 3, cod-liver oil 2, lemon juice 5, water 130.

We do not consider the composition of this paste ideal for feeding rats for an indefinite period. However, we have kept rats very easily on this paste for several months, sometimes nearly a year. We are at the present moment trying to improve the composition of this paste and to overcome the monotony of the diet.

Rats which are being used for nitrogen metabolism experiments only, so that only urine and faeces are collected, are kept in cages as described by Ackroyd and Hopkins [1916]. The food containers are placed in these cages in such a way that the rats are unable to remove them and overturn them or excrete urine or faeces into them. The glass separator of Gross and Connell [1923] and the washing of the cages were described in the paper of Chick and Roscoe [1930]. We, however, do not use dilute acetic acid for washing, distilled water giving the same results.

In order to overcome the difficulty of separating the urine and faeces of each period of the experiment accurately, sufficiently long periods of experiment are chosen. Our experience has shown that 3-day, or if possible 4-day periods are long enough since the rat excretes urine and faeces frequently. As can be seen from a sample experiment (see Table I) the data obtained are sufficiently accurate even if the periods are of two days' duration only.

The method described by Pashoutin [1886] and in this country by Haldane [1892] was used for studying the gaseous metabolism. The method is based on Pettenkofer's principle of the direct determination of carbon dioxide and moisture and indirect estimation of oxygen calculated from the changes obtained in weight of the animal and the excretion of CO_2 and H_2O. The metabolism chamber was constructed according to our design. This consists of a strong cylindrical glass jar having a flanged top edge ground on the upper surface. The lid is a thick brass plate with a rubber washer on the under side making

an air-tight joint with the edge of the cylinder. Immediately below the flange is fitted a brass ring having four swinging bolts and wing nuts which slip into slots in the lid, thus enabling it to be closed firmly. Through the lid are soldered several brass tubes one of which passes to the bottom of the jar. Through this tube the air stream passes to the bottom of the chamber thus providing perfect ventilation without allowing the rat to be in a direct draught. A zinc funnel to which a cylindrical wall is soldered, making a small cage for the rat, is placed in the lower three-quarters of the chamber. Below the tube of the funnel a small glass vessel is placed for the collection of the urine. The bottom of the zinc cage consists of two nettings resting in the funnel, an upper one of large mesh on which the rat rests and through which the faeces pass on to the under one of fine mesh. The urine passes through both nettings into the container below the funnel.

Woulff's bottles with absorbents for water (H_2SO_4) and carbon dioxide (soda-lime or KOH in sticks, moistened with water) are connected as follows, the numbers indicating the order of the bottles in the chain: No. 1 soda-lime, No. 2 KOH, No. 3 H_2SO_4, No. 4 H_2SO_4, No. 5 chamber, No. 6 H_2SO_4, No. 7 H_2SO_4, No. 8 soda-lime, No. 9 H_2SO_4, No. 10 H_2SO_4, No. 11 KOH, No. 12 H_2SO_4. The bottles Nos. 4, 7, 10, 11 and 12 are extra check bottles to indicate the completeness of the absorption in the first bottles. Bottles Nos. 1 to 4 are to remove CO_2 and H_2O from the air entering the metabolism chamber; Nos. 6 to 12 to catch H_2O and CO_2 excreted by the rat.

In the metabolism room are kept large balances, having a precision of 0·02 g., on which all the bottles and the metabolism chamber are weighed. In order to obtain accurate results the metabolism is taken for 17 hours, such a long period giving very constant figures (see Table I) covering all the fluctuations of such a small and excitable animal as the rat.

Table I. *Metabolism of a normal growing rat (per 200 g. rat per week).*

Date	Wt. of rat g.	Nitrogen g. In urine	Nitrogen g. In faeces	Nitrogen g. Balance	H_2O g.	CO_2 g.	O_2 g.
5 May	366	—	—	—	119·1	61·3	50·6
11 May	371	1·37	0·43	+0·24	106·0	62·5	51·2
12 May	371				117·9	62·4	52·0
18 May	375	1·33	0·37	+0·30	105·9	61·5	51·4
19 May	375				108·1	61·3	51·0
	Average	1·35	0·40	+0·27	111·4	61·8	51·2

Note. In this Table nitrogen metabolism was estimated for periods of 2 days only.

The usual procedure of an experiment is as follows. The rat (weighed) is placed in the nitrogen metabolism cage in the morning and food is given. A little dry boric acid is placed in the urine container. The chain is connected as given above (without the rat in the chamber) and air is drawn through for one hour before the gaseous metabolism experiment is started. The aim of the preliminary ventilation is to obtain a standardised condition of bottles and

chamber and also to remove the traces of sulphurous acid produced by fresh impregnation of pumice stone with sulphuric acid. The bottles are weighed and re-connected, the rat is placed in the chamber, which is closed during the weighing. The chamber containing the rat is weighed, after which it is connected in the chain and air passed for 17 hours. At the end of this time the chamber with the rat in it and then the bottles are re-weighed. The urine and faeces excreted by the rat while in the metabolism chamber are added to those which were excreted while the rat was at rest and feeding in the nitrogen metabolism cage so that the whole 24 hours' excreta are collected. Table I gives typical results of metabolism investigations carried out with this technique.

Before starting the experiment the amount of paste given to the rat is so adjusted that a constant weight in adult rats and only a very slow increase in growing rats is obtained; this adjustment takes about 3 weeks.

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XIX. THE VITAMIN D CONTENT OF RED PALM OIL.

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As the natural materials which contain vitamin A nearly always contain vitamin D in addition, the need of a readily available source of vitamin A free from vitamin D has at times been felt acutely in nutritional experiments [*cf.* Soames and Leigh-Clare, 1928]. It has been demonstrated [Euler, Euler and Hellström, 1928; Moore, 1929] that pure carotene can replace vitamin A in the diet, but it is not a convenient material for routine use owing to its instability. The possibility exists however that carotene may occur in some natural oil apart from vitamin D, and the present experiments were undertaken to ascertain whether red palm oil (which is relatively rich in carotene) could be used as a cheap and convenient source of vitamin A free from vitamin D.

This oil is obtained from the fruits of the oil palms growing chiefly in tropical Africa, where the best known indigenous species is *Elaeis guineensis* Jacq. The fruits of this tree are drupes, occurring in large collections called "heads[1]." Each drupe is an egg-shaped reddish brown mass of soft fibrous flesh about an inch long, enclosing the palm nut, which has a hard black shell (endocarp) containing a white kernel. It is remarkable that the flesh (or pericarp) of the fruit contains about 60 % by weight of red palm oil made up of glycerides chiefly of palmitic and oleic acids and coloured golden yellow to orange-red by the carotene it contains, while the kernel of the fruit yields the colourless palm kernel oil frequently used in compounding vitamin A-free diets, which contains 50 % of glycerides of lauric acid [Elsdon, 1926]. The palm fruit also contains lipases which become mixed with the oil when the organisation of the fruit is damaged, and commence at once to hydrolyse the glycerides of the oil, making it rancid or "hard." The rancidity of any sample of red palm oil depends therefore on the manner of its preparation from the fruit, and on its subsequent history. When red palm oil is "soft" (not rancid) it has a pleasant taste, for those who acquire it, and its rich store of potential vitamin A makes it a valuable edible oil: it is used as a culinary oil by the natives of West Africa in much the same way that butter and olive oil are employed in European countries.

[1] Certain words in this paper are enclosed in inverted commas to indicate that they are technical terms used by the producers of the red palm oil. The details given of the production of the oil are taken from many articles in the *Bulletin of the Imperial Institute*, in particular see **24**, 223 and **28**, 498.

Samples of red palm oil tested. Four specimens of red palm oil were examined, of which No. 1 was a native-rendered oil, while the others were "plantation oils," rendered by machinery in plantation factories. Some particulars concerning these oils are given in Table I.

Table I. *Properties of the four samples of red palm oil tested.*

Red palm oil sample No.	Free fatty acid content %	Lovibond yellow units per mg. of oil per cc. of chloroform solution	Lovibond blue units per mg. of oil per cc. of solution in $SbCl_3$ reagent
1	> 50	0·12	0·36*
2	3·5	1·3	0·29
3	8	1·2	0·23
4	—	2·3	0·58

* The extremely low ratio of yellow to blue colours found for this oil is at present the subject of further investigation.

The high rancidity of the native oil compared with plantation oils is due to the different methods of rendering the oil. The pericarp of the palm fruit is of firm fibrous texture and, before the oil can be extracted from it, it is necessary to soften and break down the tissues. The native method is to heap the ripe fruit into a small stone-lined pit dug in the open, sprinkle with water and leave to ferment for 2–4 weeks, at the end of which time the remaining pulpy mass is beaten daily with wooden poles to free the oil from the broken fibres of the pericarp. During this process the lipases of the fruit become mixed with the oil, and hydrolysis of its glycerides commences. It has been stated [Elsdon, 1926] that oils rendered in this way always contain 12 % or more of free fatty acids when shipped from Africa, and the rancidity increases with the age of the sample until complete hydrolysis of the glycerides sometimes takes place. On the other hand, modern factory methods of rendering aim at "sterilising" the fruit in order to destroy the lipases as soon as possible after gathering. In the most up-to-date processes the ripe fruit, carefully handled to avoid bruising, is packed into boilers and treated with steam at 30 lb. pressure for 1 hour, which serves both to destroy the lipases and break down the tissues of the pericarp. By taking this precaution to avoid hydrolysis, oils are regularly produced with a free fatty acid content of 2 %, and if especial care is taken the figure may be less than 0·2 %. Further, the rancidity of oil produced in this way does not increase as long as it is kept sealed up.

Experimental.

The four red palm oils were tested for vitamin D content by the preventive method. Albino rats at the age of 4 weeks were housed in separate cages and given the Steenbock rachitogenic diet No. 2965 [Steenbock and Black, 1925]. Each of the test animals received in addition a daily dose of one of the red palm oils from a standardised pipette delivering drops weighing between 19 and 21 mg. Four negative control animals received no addition to the basal diet, and four positive controls received a daily addition of vitamin D in 1 drop

of radiostol. At the end of 35 days the left knees of the animals were radiographed and the radiograms were examined for signs of rickets. The results are collected in Table II.

Table II. *Incidence of rickets in albino rats receiving a rachitogenic diet with the addition of daily doses of different samples of red palm oil.*

In the third column, *rickets* indicates the presence of a broad band of uncalcified osteoid tissue; *almost normal* indicates the presence of a narrow or doubtful band; *normal* means that any such uncalcified tissue was definitely absent.

Rat	Daily addition to basal diet	Radiographic finding
1 ♂	None	Rickets
2 ♂	None	Rickets
3 ♀	None	Almost normal
4 ♀	None	Rickets
5 ♀	1 drop oil No. 1	Almost normal
6 ♂	3 drops oil No. 1	Rickets
7 ♂	5 drops oil No. 1	Almost normal
8 ♂	1 drop oil No. 2	Rickets
9 ♂	3 drops oil No. 2	Rickets
10 ♀	5 drops oil No. 2	Rickets
11 ♂	1 drop oil No. 3	Rickets
12 ♂	3 drops oil No. 3	Rickets
13 ♀	5 drops oil No. 3	Rickets
14 ♀	1 drop oil No. 4	Rickets
15 ♀	3 drops oil No. 4	Rickets
16 ♂	5 drops oil No. 4	Rickets
17 ♀	1 drop radiostol	Normal
18 ♀	1 drop radiostol	Normal
19 ♂	1 drop radiostol	Normal
20 ♂	1 drop radiostol	Normal
	Further test of oil No. 1.	
21 ♀	None	Rickets
22 ♂	None	Rickets
23 ♂	3 drops oil No. 1	Rickets
24 ♂	3 drops oil No. 1	Rickets
25 ♀	5 drops oil No. 1	Rickets
26 ♂	5 drops oil No. 1	Almost normal
27 ♀	10 drops oil No. 1	Normal
28 ♂	10 drops oil No. 1	Normal
29 ♀	1 drop radiostol	Normal
30 ♂	1 drop radiostol	Normal

It is clear that, of the four oils tested, none contained a marked amount of vitamin D: indeed none of the oils afforded protection from rickets in the amounts given, with the possible exception of the native oil No. 1. A further test was therefore made with this oil (Table II). The daily doses of oil given to different animals were 3 drops, 5 drops and 10 drops respectively, and radiographic examination was made after 35 days.

Discussion.

The observations recorded above show that the native red palm oil contained more vitamin D than any of the three plantation oils tested, but that all four specimens were extremely poor in vitamin D when compared with an

ordinary cod-liver oil. The vitamin D requirements of the rat are satisfied by 3 mg. of an ordinary cod-liver oil daily [Soames and Leigh-Clare, 1928], but 100 mg. daily of the native red palm oil were insufficient to protect the rat from rickets under the conditions described. Thus the richest of the four oils used contained less than 1/30 as much vitamin D as an ordinary cod-liver oil.

The red palm oils and cod-liver oils are however almost alike in their vitamin A activity; 5 mg. daily of an average cod-liver oil supplies the necessary vitamin A for a rat, and similarly 5 mg. of a red palm oil is sufficient [Ahmad, 1931]. Therefore red palm oil may be used as a source of vitamin A free from vitamin D; in the case of the rat a daily dose of 5 mg. will ensure sufficient vitamin A while supplying a negligible amount of vitamin D. As a precautionary measure it would be advisable to use only a red palm oil of deep colour (*i.e.* particularly rich in carotene) and low free fatty acid content (*i.e.* a plantation oil).

It appears to the writer that the distinction observed between the native oil and the plantation oils may well be significant, and that native oils in general are likely to contain more vitamin D than plantation oils. This indeed is to be expected if the palm fruit contains ergosterol because the native method of preparing the oil involves leaving it exposed in the open for long periods, when irradiation by the tropical sun might well transform a small amount of ergosterol into vitamin D.

Summary.

1. Four different samples of red palm oil have been shown to contain very little vitamin D.

2. The use is recommended of red palm oil as a convenient source of vitamin A free from vitamin D in nutritional experiments, and it appears likely that any sample of red palm oil having a low free fatty acid content could be used with safety.

I wish to express my indebtedness to the Medical Research Council for a personal grant; to Dr L. J. Harris and Dr T. Moore for many suggestions and much help; and to Messrs Lever Brothers Limited, who kindly supplied the four specimens of red palm oil used in this work.

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XX. SOME PROPERTIES OF THE ENZYME GLYOXALASE.

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Technique.

In a previous paper [Giršavičius, 1931] it has been shown that, owing to a reaction of proteins with methyl- and phenyl-glyoxal, methods based on the estimation of these two substances are unsuitable for the study of glyoxalase. Reliable information about the action of this enzyme can only be obtained by studying the rate of formation of the reaction product—lactic or mandelic acid. Of the various techniques so far used for the investigation of glyoxalase only Dakin and Dudley's method of estimating mandelic acid polarimetrically [Dakin and Dudley, 1913] can be regarded as satisfactory. This method has however two disadvantages: it is rather laborious, involving four successive extractions with ether, and it is insufficiently sensitive. The second point is very important. In most enzyme kinetic studies it is essential to measure reaction velocities under conditions as nearly invariant as possible. With glyoxalase, even more than with most other enzymes, this means measuring the reaction velocity in the course of a small percentage transformation of the substrate. For the kinetics of glyoxalase are complicated by two factors: the non-enzymic reaction in which the substrate is rapidly destroyed, and the production of an acid from a neutral substance by the action of the enzyme.

To shorten the necessary reaction time, and yet obtain sufficiently large polarimetric readings, the volume to which the mandelic acid has to be made up for polarimetry has been diminished in the present work by using 2 dm. polarimeter tubes, holding 3–3·5 cc. Further, the rotation of the mandelic acid has been increased by the addition of uranyl nitrate. As discovered by Walden [1897] uranyl nitrate increases the rotation of mandelic acid 2·5-fold under suitable conditions. In the present experiments a 10 % solution of uranyl nitrate (12·75 g. $UO_2(NO_3)_2$, $6H_2O$ per 100 cc.) in 1 % NaOH was used. The mandelic acid solution is evaporated down, the residue is taken up with 4·5 cc. uranyl nitrate solution and filtered. The rotations obtained under these conditions are not directly proportional to the quantities of *l*-mandelic acid used, but increase less rapidly. In Table I some figures are given, from which a calibration chart can be constructed, and corrections, to be applied to the readings, obtained.

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Table I.

l-Mandelic acid solution cc.	Rotation
5	− 2·37°
5	− 2·32°
5	− 2·34°
10	− 4·46°
10	− 4·38°
25	− 10·08°
25	− 10·16°

Taken up in 4·5 cc. water, after evaporation, 5 cc. of the solution gave a rotation of − 0·98°. The rotation is thus found to be increased 2·40 times at a concentration corresponding to a rotation of − 1° in water. The increase at other concentrations can be calculated by means of the above table, since the rotation in water increases linearly at the dilutions here considered. In these experiments, as well as in the following ones of this paper, the polarimetric readings refer to the yellow line of the Hg spectrum ($\lambda = 577$). Using the green line ($\lambda = 546$) a rotation increase of 2·50 (at − 1°) is obtained; the rotation in water is itself larger at the lower wave-length. Nevertheless the yellow line was used in these experiments, the light of the green line being absorbed more strongly by the solutions used. Only in the following, earlier, experiment were the readings made at $\lambda = 546$.

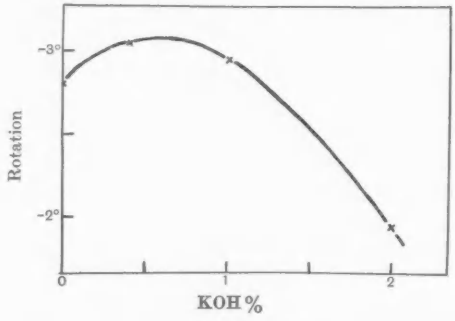

Fig. 1.

In Fig. 1 the results of an experiment on the rotation of mandelic acid in a uranyl acetate solution with varying quantities of alkali are recorded. This experiment was done before the present technique had been worked out, and the experimental conditions are different. It shows however that a 2·5-fold increase in rotation is about maximal. 5 cc. of a *l*-mandelic acid solution (the *l*-mandelic acid used in these experiments was prepared from amygdalin) with varying additions of 10 % KOH were made up to 50 cc. Excess of solid uranyl acetate, which according to Critical International Tables is soluble to 7·8 %, was added. After standing overnight the solutions were filtered and the

rotations read in a 4 dm. tube. The same quantity of mandelic acid gave in 50 cc. water a rotation of − 1·21°.

The extraction method has also been modified, so as to diminish the number of extractions required, and at the same time obtain more nearly colourless extracts. The last point is specially important with the semimicro-polarimetry used in this work. At the time when lactic acid extraction used to be an integral part of lactic acid estimation this process had been markedly improved by the substitution of amyl alcohol for ether [Ohlsson, 1916]. To see whether a similar improvement was possible in the estimation of mandelic acid some experiments were made on its distribution between amyl alcohol and concentrated ammonium sulphate (acidified with H_2SO_4). A partition coefficient of about 28 was found[1], so that a single extraction with three times the volume of amyl alcohol should remove 98–99 % of mandelic acid from an ammonium sulphate solution. From the amyl alcohol the mandelic acid can be recovered by extracting twice with 20 cc. of 0·2 *N* NaOH. This leaves about 1–1·5 % mandelic acid in the amyl alcohol, as shown by the following experiment (among others). Varying quantities of mandelic acid solution had been extracted with 300 cc. lots of amyl alcohol. After running out the aqueous layers and washing the walls and stems of the separating funnels with 20 cc. of water, the amyl alcohol layers were shaken twice with 20 cc. lots of NaOH (about 1·5 mins. each time), then washed with 10 cc. water. The two NaOH extracts and the last washing were collected together as *a*. The process was repeated with one shaking with NaOH and one washing: *b*. (1 *a*) − 3·72°; (1 *b*) − 0·06°; (2 *a*) − 6·89°; (2 *b*) − 0·09°.

Irregular results were at first obtained, which turned out to be due to the effect of small amounts of ammonium sulphate solution clinging to the walls of the separating funnel and passing into the NaOH extract. Ammonium sulphate diminishes the rotation of mandelic acid in uranyl nitrate solution. These remnants of ammonium sulphate are removed by washing with 10 cc. of water before extracting with NaOH. The partition coefficient of mandelic acid between amyl alcohol and water has been found to be about 3·8, so that 10 cc. of water would remove less than 1 % of mandelic acid from 300 cc. amyl alcohol.

The extraction is now carried out as follows. 100 cc. of the ammonium sulphate solution, containing the mandelic acid, are pipetted into 300 cc. amyl alcohol (coloured with Sudan III to emphasise the boundary) in a 500 cc. separating funnel. This is shaken for 2–3 mins. After separation the aqueous layer is run out. By a circular shake drops of the aqueous solution clinging to the walls are forced to the bottom; this is also run out. Now 10 cc. water are pipetted into the funnel, which is briefly shaken. After the water has been run out the stem of the funnel is rinsed with a jet of water. 20 cc. 0·2 *N* NaOH are pipetted into the funnel, which is shaken for about 1½ mins. This process is repeated. The walls of the funnel are now washed with 20 cc. of water. The two

[1] No great accuracy is claimed for this figure, as the mandelic acid in the aqueous phase could not be determined directly, but had to be estimated as the difference between initial and final concentration in the alcohol, that is, as the small difference between two relatively large figures.

NaOH extracts and the final washing are run into a 3 in. crystallising dish. Between the extractions the stem of the funnel is washed out with a jet of water into the same dish. To the joint extracts about 1 cc. of 0·1 % aqueous solution of *p*-nitrophenol is added and 5 cc. *N* H_2SO_4. The neutralisation is finished with 0·2 *N* H_2SO_4. (Because of the high CO_2 content of the solutions it seems more convenient to over-acidify them at first, and finish the neutralisation with CO_2-free NaOH and 0·2 *N* H_2SO_4 after partial evaporation.) The solutions are evaporated on a boiling water-bath under a current of air. The dry residues are treated with 4·5 cc. uranyl nitrate solution, filtered, and the rotations are read as described above. The mandelic acid is not racemised during the evaporation, nor is its rotation affected by the Na_2SO_4 formed in the neutralisation. The following experiment illustrates the method. Samples of 10 cc. *l*-mandelic acid solution + 90 cc. concentrated ammonium sulphate (acidified) were treated as described. Readings: − 3·27°; − 3·38°; − 3·25°. 10 cc. of the solution neutralised and evaporated directly gave − 3·51°. 94 %, with a maximum deviation of ± 2·4 %, were recovered in these experiments.

For the estimation of mandelic acid in liver extracts Dakin and Dudley's method of deproteinising was used, in a slightly modified form. To 100 cc. of the liver extract 5 cc. of 60 % (by volume) H_2SO_4 were added and 70 g. of solid ammonium sulphate. After heating on a water-bath the solutions were made up to 150 cc. with concentrated ammonium sulphate solution and filtered. 100 cc. of the filtrate were treated as described. Very surprisingly it has been found that this method of deproteinising causes a considerable loss of mandelic acid. Details will not be reported here as the matter is still under investigation; in a fairly extensive series of experiments a recovery of 76·8 % of *l*-mandelic acid added has been found, with a probable error of ± 4·2 %.

Enzyme experiments.

In the following experiments the above technique has been used for the estimation of mandelic acid. The liver extracts used were prepared by shaking minced ox liver (fresh from the butcher) for an hour on the shaking machine with five times the weight of water, then filtering through muslin. The extracts were stored in the refrigerator and generally used 12 hours after they had been prepared. The first experiment deals with the effect of toluene on glyoxalase. Dakin and Dudley [1913] have stated that glyoxalase action is inhibited by toluene. Kuhn and Heckscher [1926], using methylglyoxal, and estimating the rate of disappearance of the substrate, have come to the opposite conclusion. They think that the inhibition in Dakin and Dudley's experiments may have been due to extraction of phenylglyoxal from the aqueous solution by the toluene.

Solution (1): 50 cc. liver extract, 20 cc. 10 % $CaCO_3$ suspension, 20 cc. water, 10 cc. phenylglyoxal solution (about 15 mg. per cc.).

Solution (2): like (1) but shaken with 2 cc. toluene before adding the phenylglyoxal.

Both solutions were incubated for 5½ hours at 37°. Readings: (1) − 2·70°; − 2·64°. (2) − 1·96°; − 2·04°. There is an inhibition of 20·5 %. This can scarcely be accounted for by extraction of the substrate, but the question needs further investigation. All subsequent experiments were done without addition of an antiseptic. Dakin and Dudley state that phenylglyoxal itself acts as a mild antiseptic. In the present experiments no obvious signs of putrefactive changes were present after incubation.

The next point that needed investigation was the effect of suitable buffer substances on the activity of glyoxalase. According to Kuhn and Heckscher glyoxalase has a p_H optimum at about 8·2. At this p_H borate and phosphate buffer can be used (though with little buffering power), also the diethyl barbiturate buffer recently introduced by Michaelis [1931]. In the following experiment the activities of the enzyme in presence of various concentrations of these buffer-substances are compared.

Table II.

	Liver extract cc.	Na barbitone cc.	Water cc.	Phenyl-glyoxal cc.	Initial p_H	Final p_H
1	140	175	—	35	8·10	8·03
2	140	75	100	35	8·10	7·83
3	140	35	140	35	8·02	7·56
		Phosphate				
4	140	175	—	35	—	7·87
5	140	75	100	35	8·05	7·79
6	140	35	140	35	7·98	7·66
		Borate				
7	140	175	—	35	—	7·85
8	140	75	100	35	—	7·75
9	140	35	140	35	7·88	7·37

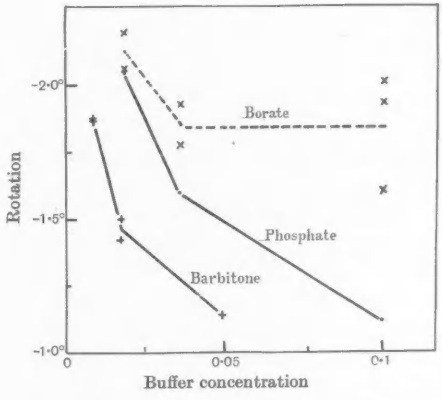

Fig. 2.

The solutions were incubated for 16½ hours at 26·5°. The diethyl barbiturate (barbitone) buffer was 0·070 *M* in barbitone, the phosphate buffer (Sørensen) was 0·2 *M* in PO_4, the borate buffer (Palitzsch) 0·2 *M* in BO_2. The results obtained are plotted in Fig. 2. On the abscissa the *M* buffer concentrations are plotted, in the terms just described. Unfortunately they are not strictly comparable in this way, which assumes that each buffer solution contains only one molecular species. In fact the diethyl barbiturate buffer contains both the undissociated acid and its ion, the phosphate buffer contains both HPO_4'' and H_2PO_4' ions, whilst in the borate buffer several, partly polymeric, molecular and ionic species are present. How much of the inhibition found is due to one or other ion or molecule in the solution cannot be decided without further investigation, so that the inhibitions produced by the various buffers cannot be compared exactly. It appears however that borate has little inhibiting action, phosphate inhibits the enzyme markedly, whilst diethyl barbiturate has a strong inhibiting action. That glyoxalase is inhibited by phosphate has been shown by Sakuma [1930]. It is remarkable that an enzyme, which presumably is a part of the glycolytic system, should be sensitive to phosphate. When

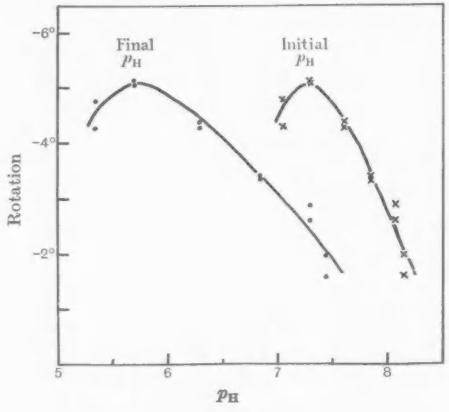

Fig. 3.

extracting the solutions containing borate it was found that boric acid follows mandelic acid through the extraction process. As no way could be found of separating the two substances, and as boric acid diminishes the rotation of mandelic acid, the following artifice was used. The final uranyl nitrate solutions containing mandelic acid and some boric acid were saturated with solid H_3BO_3. The diminution in rotation so produced was determined separately, and corrected for. The results, however, are not as consistent as with the other solutions. Although diethyl barbiturate inhibits more strongly than the other buffers, its greater buffering power in what was believed to be the optimum p_H range of glyoxalase led to its being used in the following experiment on the

p_H-activity curve of the enzyme. The solutions consisted of 100 cc. liver extract, 30 cc. diethyl barbiturate solution (0·1 *M*), 25 cc. phenylglyoxal solution, enough 0·1 *N* H_2SO_4 to give the desired p_H, and enough water to bring the volume up to 250 cc. (The quantities of H_2SO_4 to be added were calculated on the basis of the titration curve of the buffer (Michaelis). In fact the actual initial p_H values of the mixtures came out considerably lower than intended, owing, apparently, to the buffering power of the neutral liver extract.) The solutions were incubated for 17½ hours at 26·5°. The p_H was again determined at the end of the incubation. As Fig. 3 shows, a very considerable fall in p_H has taken place during the reaction. The optimum seems to lie between p_H 6 and 7; its exact position and the shape of the p_H-activity curve of glyoxalase cannot, of course, be deduced from these figures. The difference of the optimum p_H range found in this experiment from that reported by Kuhn and Heckscher must be at any rate partly due to the fact that these authors only determined the initial p_H in their experiment. The fact that they used phosphate buffer, with little buffering power at the higher p_H values, must also have contributed to the discrepancy. On the other hand part of the shift into the acid range observed in this experiment, as compared with Kuhn and Heckscher's observations, may of course be due to a specific action of the buffer used. This will have to be further investigated.

Summary.

1. A method is described for estimating active mandelic acid in tissue extracts, by extracting with amyl alcohol and enhancing the rotation with uranyl nitrate.

2. By means of this method the effect of toluene and of various buffer substances on glyoxalase has been investigated, as well as the p_H-activity curve of the enzyme.

My thanks are due to Sir F. G. Hopkins and to Prof. J. B. S. Haldane, for their kindness and encouragement.

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XXI. A NOTE ON DENATURED GLOBIN.

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The formation of a protein material insoluble at the isoelectric point in a solution of native protein is known as denaturation, and the protein is said to be denatured. The physical criterion of insolubility has been widely applied to decide whether a protein has been denatured or not by some extraneous influence. Besides this physical test the formation of an —SH (or of an —S—S—) group may likewise be used; this latter standard has the advantage that it can be applied to the changes undergone by keratins where solubility alterations cannot be studied. When the nitroprusside test of Mörner [1901] is applied to denatured proteins it is found that these can be divided into three classes:

(1) those which give a reaction for an —SH group;

(2) those which give a reaction for —S—S—;

(3) those which do not give a reaction for either free —SH or —S—S—.

Egg-albumin [Heffter, 1907] typifies the first group, and serum-proteins the second. Ovomucoid [Harris, 1923] and globin [Meldrum and Dixon, 1930] yield neither sulphydryl nor disulphide groups and accordingly belong to the third class.

Anson and Mirsky [1931] state that Meldrum and Dixon's observation is erroneous and claim to have obtained a preparation of denatured globin which gave tests for both —SH and —S—S— groups.

The author has re-examined the matter: he has confirmed the original observations of Meldrum and Dixon [1930], and has succeeded in explaining those of Anson and Mirsky. Ox and sheep globins were prepared by three different methods:

(1) the Schulz [1898] method,

(2) according to Hill and Holden [1926],

(3) by the acetone method of Anson and Mirsky [1930].

The first two methods consist, in essence, in removing haematin from globin by the use of ether, the aqueous layer being acid. The last process consists in precipitating free globin by adding excess of acid acetone to an acidified solution of carboxyhaemoglobin. Schulz's method yields entirely denatured globin, the others a mixture of denatured and native material[2].

Globin preparations having been made in these three ways, the acid aqueous

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[2] It is best to use haemoglobin dialysed free from the glutathione present in blood-corpuscles.

solutions were boiled, or saturated with urea, and allowed to stand. The denatured material was ground moist with ammonium sulphate and sodium nitroprusside, and alkali was added. The test was negative with globins made by the methods of Schulz and of Hill and Holden. Thus the sulphydryl group appears to be absent from denatured globin made by the use of ether. In the case of acetone-globin a faint purplish tint appeared. This differed from the typical nitroprusside test for —SH in being of a different colour. It was more stable and did not disappear with time as does the typical nitroprusside reaction for —SH. When globin made in these three ways was ground with $(NH_4)_2SO_4$ and potassium cyanide the nitroprusside test was again negative with ether-globin, and again a faint purplish tint appeared with acetone-globin, identical in appearance with that obtained before treatment with cyanide. The statement of Anson and Mirsky that denatured globin contains the normal amount of —S—S— groups could not be confirmed.

Now the Rothera test for acetone bodies resembles the nitroprusside test for sulphydryl closely. Grote [1931] has described a method by which the nitroprusside test for —SH can readily be distinguished from that due to acetone. When the solution to be tested is saturated with sodium bicarbonate and sodium nitroprusside added the typical colour is developed when sulphydryl is present, but acetone bodies do not react. On applying this test to acetone-globin no colour develops, but the typical purple appears when ammonia is used as alkali.

The following experiment was devised to analyse the system further. A 5 % solution of acetone-globin was slowly neutralised as described by Anson and Mirsky [1931]. The precipitate of denatured globin yielded a pale purple colour with the nitroprusside reagent in the presence of ammonia and ammonium sulphate, but not in saturated bicarbonate. The solution of native globin obtained by filtration from the denatured fraction was electrodialysed for 8 hours in the apparatus of Stadie and Ross [1926], the current density being 0·2–0·3 amp. A solution of native globin thus obtained yielded no observable nitroprusside reaction after it had been denatured by boiling or treatment with urea.

Summary.

1. The observation of Meldrum and Dixon [1930] that denatured globin does not yield a nitroprusside reaction for free —SH or —S—S— groups has been confirmed.

2. The statement of Anson and Mirsky [1931] that denatured globin contains the amount of —SH and —S—S— groups normal to most denatured proteins could not be confirmed.

3. Only in the case of globin prepared with acetone does any colour appear on treating with nitroprusside. This colour differs from the normal given by sulphydryl, and does not appear when the conditions are chosen to obviate reaction between nitroprusside and acetone.

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XXII. BILIRUBIN AND THE VAN DEN BERGH REACTION.

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Introduction.

The difference between the bile pigment which gives the "direct" van den Bergh reaction and that which gives only the "indirect" reaction has been the subject of much investigation, and many attempts have been made to explain it; none of them however appears to be entirely satisfactory.

Davies and Dodds [1927] have reviewed this work up to the end of 1926, at the same time offering a contribution of their own in which they suggest that the pigment of blood-sera giving the direct reaction is bilirubin, while that of sera giving only the indirect reaction is an oxidation product of bilirubin. Roberts [1928] put forward the view that bilirubin in the free colloidal condition was the pigment of indirect sera, while in direct sera the bilirubin was in combination with some substance the nature of which is as yet undetermined. Newman [1928] opposed the view that the pigment of indirect sera was free bilirubin and claimed that the pigment of direct sera was sodium hydrogen bilirubinate. M'Gowan [1930] considered that the pigments of direct and indirect sera were identical, the types of van den Bergh reaction being dependent on the alkali reserve of the blood; indirect reactions were obtained when the alkali reserve was diminished. Snider and Reinhold [1930] claimed that the type of van den Bergh reaction was dependent on the concentration of the pigment which is present, direct reactions being associated with high concentrations of bilirubin, indirect reactions with low concentrations. They also called attention to the accelerating effect of alkali, which has been noticed by other workers. Griffiths and Kaye [1930] agreed with Roberts in ascribing the indirect reaction (in sera giving this reaction alone) to free bilirubin, but they stated that "direct-reaction pigment" was not bilirubin or one of the ordinary salts of bilirubin. It should be added that the three papers last mentioned appeared after the commencement of the work about to be described.

Collinson and Fowweather [1926] put forward the view that the pigment of indirect sera was free bilirubin, while the pigment of direct sera was a salt of bilirubin, probably the ammonium salt. It will thus be seen that our views on

the nature of the indirect pigment have been supported by Roberts and by Griffiths and Kaye, while our view regarding the direct pigment, so far as it considers this to be a salt of bilirubin, is supported by Newman though he is not in agreement with us as to the probable nature of this salt.

Many of the experiments described by some of the workers quoted above appear to support the view that the difference between the pigments concerned depends on whether we are dealing with a free acid or a salt. Newman, for example, showed that the addition of alkali in proper amounts to indirect sera would convert the reaction to a direct one, while the addition of acid would convert a direct to an indirect one. Experiments of this nature however do not prove the point conclusively, for while it may be accepted that a bile-pigment-containing serum to which alkali has been added probably contains a salt of bilirubin, the serum will be more alkaline than a natural serum which gives a similar result with the van den Bergh reagent. The conditions under which the reaction takes place are decidedly different in the two cases, and therefore the fact that identical results are obtained hardly allows us to assume definitely that the pigments responsible for these results must be identical, though a similarity and possibly an identity is suggested. Another objection to experiments of this type is that they do not, in all probability, represent what actually occurs in the body. The two types of pigment, according to the theory most generally accepted at the present time, do not alter after their introduction into the blood; their production and any change that occurs from one type to another take place in certain organs of the body and not within the blood stream.

It appeared to us therefore that more valuable evidence would be obtained by preparing and isolating in the solid state certain salts of bilirubin and noting what type of reaction was obtained when small amounts of these were added to normal serum.

Methods.

The bilirubin we have used has been prepared from human gallstones. These were finely ground and extracted with chloroform to remove all cholesterol. The residue was well washed with water and then treated with dilute hydrochloric acid, a small amount of sodium sulphite being added to minimise oxidation. The resulting product after washing and drying was again extracted with chloroform. The chloroform extract, as it became concentrated, began to deposit brick-red crystals of bilirubin, and after a quantity of these had been formed the extract was filtered, and the crystals were washed with a little cold chloroform.

The filtrate and washings were treated with excess of light petroleum, which caused the precipitation of a yellowish brown amorphous solid. This was also filtered and washed with light petroleum.

Most of the experiments to be described were performed with the red,

crystalline bilirubin; a few, however, as will be indicated later, were carried out on the precipitated product.

The van den Bergh reaction was carried out only in the direct form, reagent being added to serum in the proportion of 0·4 cc. of reagent to 0·5 cc. of serum.

Experimental.

Preparation of certain salts of bilirubin, and their reactions when added to serum.

The preparation and analysis of the mono-ammonium salt are given by Küster, Reihling and Schmiedel [1914]. The product obtained is dark brown in colour, very soluble in water, but also soluble in cold chloroform; it only gave a delayed reaction in serum, in agreement with Grünenberg's [1922] observation that the pigment of sera which give a prompt direct reaction is not extracted by chloroform.

An attempt was next made to prepare a monosodium salt by a method analogous to that used for the ammonium salt. A weighed quantity of bilirubin was suspended in methyl alcohol and the theoretical quantity of 0·1 *N* sodium ethoxide was added. Only a portion of the bilirubin went into solution, and so a further equivalent of sodium ethoxide was added. After shaking, and rubbing the coarser particles, the whole of the solid originally present dissolved. The solution was precipitated with ether and the precipitate was filtered off and dried. (Found: Na 7·42 %; the disodium salt requires 7·32 %.)

This, product when dissolved in serum only gave a delayed reaction.

A further attempt was made to prepare the mono-salt. Bilirubin was again suspended in methyl alcohol, and one equivalent of sodium ethoxide was added. Some bilirubin went into solution immediately, but after a few minutes there appeared to be a deposition of solid from the solution, suggesting that the di-salt was first formed but later reacted with some of the free bilirubin present, to form the mono-salt. The mixture was left in an ice-chest overnight and next morning much of the solid seemed to have disappeared. The solution was filtered from a small amount of residue which appeared to be free bilirubin. The filtrate was treated with ether and the resulting precipitate isolated and dried. (Found: Na 3·88 %; monosodium bilirubinate requires 3·80 %.)

This, too, when dissolved in serum gave a delayed reaction.

In another preparation a considerable excess of sodium ethoxide over that required to form the di-salt was used. Again only a delayed reaction was obtained when the resulting product was added to serum.

In a further series of experiments water was used in place of methyl alcohol. A weighed quantity of bilirubin was suspended in water and one equivalent of 0·1 *N* sodium hydroxide added. Again much of the bilirubin failed to dissolve, and neither the remaining solid nor the filtrate from it, nor a mixture of the two, gave a prompt reaction when added to serum. Two equivalents of alkali caused all the bilirubin to dissolve, but addition of the solution

to serum gave the same result as before; addition of further equivalents, one at a time until ten equivalents had been added, produced the same result.

Experiments were next made to ascertain the effect of p_H on the preparation. A series of twelve solutions was prepared from B.D.H. universal buffer, starting with a p_H of 7·0 and advancing by 0·5 up to 12·5. To a little solid bilirubin 3–4 cc. of the solution were added, and the mixture was well shaken and allowed to stand for half an hour at room temperature. On inspection, no solution of the bilirubin was apparent at p_H 7·0 to 10·5. Partial solution was evident at p_H 11·0, and complete solution at p_H 11·5 to 12·5. After centrifuging, the supernatant fluid was practically colourless with solutions of p_H 7·0 to 8·0, slightly but increasingly yellow at p_H 8·5 to 10·5, but deep red at p_H 11·0 and upwards. When 0·1 cc. of the supernatant fluid in each case was added to 0·5 cc. of serum and the van den Bergh reagent added, very little change was produced with solutions at p_H 7·0 to 10·5, whereas with the remainder some colour production occurred on the addition of the first few drops of reagent, but full colour development was very slow. This series of experiments was then repeated, the mixtures being kept at 37° for 3 hours. This modification however also failed to produce a product which would give a prompt direct reaction when added to serum.

Preparation of a direct-reacting pigment from bilirubin.

Bilirubin is readily soluble in the cold in dilute solutions of sodium and potassium carbonates, and when one or two drops of such solutions are added to 0·5 cc. of serum the resulting mixture gives a biphasic reaction. In such cases the conditions are similar to those of Newman's experiments, in which the serum mixture was decidedly more alkaline than a natural serum. With bicarbonates the same objection does not hold. Bilirubin is not soluble in the cold in solutions of either $NaHCO_3$ or $KHCO_3$, but dissolves on warming, and when the solutions are added to serum biphasic reactions are obtained. Other salts which in aqueous solutions show an alkaline reaction were next tried, *e.g.* K_2SO_3, Na_2SO_3, Na_2HPO_4 and K_3PO_4. The nearest approach to a prompt direct reaction was obtained with K_3PO_4 and further experiments were made with it. In a moderately strong solution bilirubin is not completely soluble. Hence the mixture was centrifuged and 1 drop of the supernatant fluid (in which rapid oxidation of the pigment on standing was noticed) was added to serum. This gave a reaction in which colour development began rapidly and reached its maximum in about 5 minutes. The deposit left after removal of this fluid gave a much slower reaction. A stronger solution of K_3PO_4 was next tried, but in this bilirubin was insoluble; in a dilute solution bilirubin dissolved completely, but the solution only gave a delayed reaction when added to serum. It was then found that if to a complete solution of bilirubin in dilute K_3PO_4 a strong solution of the salt is added, the pigment is almost completely precipitated. Such a mixture was centrifuged and a little of the precipitate was added to serum, in which it was readily soluble. The serum was then found to give a typical

prompt reaction. If, however, the precipitate were first dissolved in water and some of this solution added to serum, no prompt reaction was obtained. The precipitate itself appears to be insoluble in cold chloroform; in aqueous solutions it oxidises rapidly.

Having obtained a method for the preparation of a direct-reacting pigment from bilirubin, various factors in the method were examined separately and the following results obtained.

(*a*) It is not necessary to dissolve the bilirubin in a weak solution of the salt whose concentrated solution is the precipitating agent. Any of the alkaline substances capable of bringing bilirubin into aqueous solution may be used for the preparation, including the caustic alkalis. A convenient method is to suspend the bilirubin in water and add just sufficient dilute ammonia to bring about complete solution of the pigment.

(*b*) K_3PO_4 is not the only precipitating agent which can produce the desired result. Most of the alkaline phosphates can be used, *e.g.* K_2HPO_4, Na_2HPO_4, $(NH_4)_3PO_4$. It was found too that ammonium carbonate was quite as good in this respect as any of the phosphates. Sodium bicarbonate was not quite so good, *i.e.* the reaction obtained with the precipitate was slightly delayed. Potassium acetate was not so good as sodium bicarbonate, while sodium sulphite was useless.

Caustic alkalis themselves may be used as precipitating agents. Thus the addition of concentrated sodium hydroxide to bilirubin dissolved in a dilute solution of the same reagent brings about precipitation of the direct-reacting pigment.

In strong ammonia bilirubin is at first soluble, but soon a gelatinous precipitate forms which shows direct-reacting properties.

(*c*) Neutral or acid salts cause precipitation of a pigment in the same way as do the alkaline salts just mentioned, but the precipitate obtained is not a direct-reacting pigment.

It thus appears that a direct-reacting pigment is obtained as a precipitate when concentrated solutions of alkalis or of certain alkaline salts are added to solutions of bilirubin in dilute alkalis or in dilute solutions of these alkaline salts.

The isolation of this direct-reacting pigment as a pure, dry solid has not yet been accomplished; the tests of the product have been made with the wet solid left behind after centrifuging and removing as far as possible the clear supernatant layer. The solid when precipitated is somewhat gelatinous though it is easily separated by centrifuging; filtration is very slow, and during filtration and drying certain changes occur, so that the resulting dry solid fails to give a prompt reaction when dissolved in serum. A dried preparation containing the direct-reacting pigment has however been prepared, and will be referred to later.

It was shown by the following experiment that the ability to give the prompt reaction still remains after removal of the precipitating solution.

Bilirubin, dissolved in a little ammonia, was treated with saturated ammonium carbonate solution. The precipitate was centrifuged and washed in the centrifuge-tube with saturated sodium chloride solution. The wet solid still gave a prompt reaction when added to serum. Filtration was attempted, but after being on the filter for some time a portion of the solid, still moist, only gave a delayed reaction when added to serum.

Experiments with Griffiths and Kaye's direct-reacting pigment.

At this stage of the investigation Griffiths and Kaye's communication appeared. In this the isolation of a direct-reacting pigment (in an impure state) from bile is described, and the question as to the possible identity of this pigment with the one prepared from bilirubin by the above method seemed to be of considerable importance. From the treatment accorded to the pigments in each case, identity seemed extremely unlikely. The method of preparation of the direct-reacting pigment from bilirubin makes it certain that the product is a salt of some kind, whereas the isolation of the direct-reacting pigment from bile by the method of Griffiths and Kaye, in which the bile is made acid to Congo red with hydrochloric acid, suggests that the extracted pigment is a free acid. In the latter method, however, it appeared possible that some salt present in the bile might be occluded by the thick, slimy precipitate which at once forms on addition of hydrochloric acid, and so escape further action of the acid and be responsible for the direct-reacting properties of the final product. It was therefore decided to ascertain if a direct-reacting salt could be obtained from bile if treatment by hydrochloric acid is omitted.

A quantity of human bile was evaporated to dryness *in vacuo*, and the sticky residue was washed with light petroleum to remove fatty matter. The solid was then ground up and extracted with ether in the apparatus used for extracting cholesterol from blood [Myers and Wardell, 1918]. On evaporation of the ether a small amount of yellow solid was left, which, on addition to serum gave a prompt reaction. The residue from the ether extraction was then extracted with chloroform. Much more pigment was extracted by the chloroform than by ether and, after evaporating off the chloroform in a current of coal-gas, a considerable quantity of a greenish brown solid remained. On addition of a small amount of this solid to serum an excellent prompt reaction was obtained. The solid after ignition left a soluble, fusible alkaline ash which contained sodium. A solution of the solid in water, when added to serum, also gave a prompt reaction, but not quite so good as that obtained with the solid itself.

The method of isolation of this direct-reacting pigment without the use of any acid, its solubility in water, and the nature of the ash, give strong grounds for supposing that the product is a sodium salt. On treatment of its aqueous solution with hydrochloric acid a precipitate is formed.

The result of this experiment does not conclusively prove that Griffiths and Kaye's direct-reacting pigment is also a sodium salt.

A fresh specimen of bile was diluted with two or three volumes of water and the well-stirred mixture made acid to Congo red with hydrochloric acid. The precipitate was washed with water and then dried *in vacuo*. It was then ground and washed with light petroleum and extracted first with ether and then with choloroform.

The ether extract after evaporation left a yellowish brown solid which was insoluble in water but soluble in serum, in which it gave a prompt reaction. The chloroform extract yielded a greenish solid which showed the same solubility and reaction as the ether extract. On igniting the ether extract no ash whatever remained, and from the chloroform extract only a very slight trace of ash was left. No ammonia was present, so that it was thought that the substance must be a free acid. Its general behaviour was similar to that of the red bilirubin extracted from gallstones, but with the outstanding difference that it was soluble in serum and when so dissolved gave a prompt reaction. It was also found to be soluble in the cold in sodium bicarbonate solution. The ether-extracted portion also resembled gallstone bilirubin in colour, except that it was somewhat yellower.

The results so far obtained show that not one but two direct-reacting pigments may be isolated from bile, their probable relationship being that of free acid to sodium salt.

It should be added that not all specimens of bile yield direct-reacting pigments when treated by the methods outlined above. Only fresh, golden bile can be relied on to give these results; old or very dark coloured specimens generally yield pigments which on addition to serum give biphasic or delayed reactions. It is evident therefore that the direct-reacting pigments are somewhat unstable. The loss of direct-reacting property is not entirely due to oxidation though this certainly occurs. It must be due in part to a change to a more stable, indirect-reacting pigment.

In the above experiments, though extractions have been made with both ether and choloroform, it is not considered that there is any essential difference between the two extracts. In both cases the pigments are accompanied by other substances extracted from the bile. The amount of material obtained with chloroform is always greater than with ether, but with chloroform more oxidation of the pigment occurs owing to the higher temperature of extraction, and this no doubt accounts for the differences in colour which have been noted.

The experiments described have shown that the direct-reacting pigment prepared from bilirubin is not identical with Griffiths and Kaye's pigment, since the latter is not a salt. There remains the possibility that it is identical with the direct-reacting pigment obtained from bile without acidification, and attention was next directed to this point.

Between the two pigments under consideration there is one important difference, namely, that the pigment prepared from bilirubin loses its direct-reacting properties if dissolved in water, but the same is not true, at any rate to the same extent, of the material isolated from bile. But the latter contains

other substances from bile besides the pigment, and even this has some tendency to change to a non-direct-reacting form, as shown by the fact that old specimens of bile tend to yield a product without direct-reacting properties. The difference in stability is relative, and it appears possible that the greater stability of the bile product may be due to the effect of the substances accompanying the pigment. An attempt was therefore made to separate the latter from the other substances present. Concentrated sodium hydroxide solution (in which the direct-reacting pigment from bilirubin is insoluble) was added to an aqueous solution of the direct-reacting product from bile until a precipitate was formed. On centrifuging a pellicle of brown pigment resulted, lying on top of a practically colourless solution. This pigment gave a prompt reaction when dissolved in serum, but if previously dissolved in water and its solution added to serum only a delayed reaction was obtained, *i.e.* it behaved like the pigment from bilirubin. This result suggests either that the pigment from bile is identical with that from bilirubin but is stabilised by other substances accompanying it and which have been separated from it by the procedure adopted in this experiment, or that the two pigments are closely related and that the pigment from bile is converted to a form identical with that from bilirubin by the treatment described.

Bile salts are among the substances present in the direct-reacting product from bile, and experiments were made to determine the stabilising effect of these on the direct-reacting pigment prepared from bilirubin. In one of these bilirubin, suspended in water, was dissolved by the aid of a little ammonia, and sodium glycocholate was added. The resulting solution was not precipitated by strong ammonium carbonate solution, and did not give a prompt reaction when added to serum. When a little more ammonia was added, however, it did give a prompt reaction. In the presence of bile salt, therefore, the direct-reacting pigment is formed by a sufficient concentration of alkali, without precipitation of the pigment. The solution was evaporated to dryness *in vacuo*, but the resulting solid was without direct-reacting properties.

In another experiment bilirubin in dilute potassium hydroxide was precipitated with tripotassium phosphate and the precipitate separated by centrifuging. The supernatant fluid was poured off and replaced by a solution of sodium glycocholate, in which the precipitate dissolved. The solution, however, did not give a prompt reaction until a little more tripotassium phosphate had been added. After doing this the solution was added to an equal volume of serum and the mixture evaporated to dryness *in vacuo*. The dried residue was yellowish-red, and when added to serum gave a prompt reaction. When dissolved in water it was alkaline to litmus and when treated with chloroform in the extraction apparatus very little pigment was extracted by the solvent. This solid represents the first successful attempt to obtain a direct-reacting pigment prepared from bilirubin in the solid state, but it differs in its alkaline reaction and solubility in chloroform from the direct-reacting material obtained from bile.

In another case the direct-reacting pigment was prepared and centrifuged in the usual way and then dissolved in about 10 cc. of serum. It was then divided into two parts. The first part was evaporated *in vacuo*. To the second sodium glycocholate was added, and then it was also evaporated. The solid from the first part, when dissolved in serum, gave a reaction which was fairly rapid, but not prompt, if it was in fair concentration, but much less rapid if in dilute solution. The solid from the second part gave a prompt reaction in fairly concentrated solution, less prompt when dilute.

Here there does seem to be an increased stability, so far as direct-reacting properties are concerned, in the presence of bile salts, but neither in this nor in many other experiments with bile salts was a product obtained having the same stability as that obtained from bile.

The identity of the pigment from bilirubin with that isolated from bile has therefore not so far been proved, but the evidence suggests at any rate that it is highly probable. Bile salts may not be the sole or even principal stabilising agents, and other substances in the bile extract which might act in this way have not so far been examined.

Küster's two forms of "Bilirubinammonium."

The simplest and most satisfactory explanation of the facts so far recounted is that bilirubin may exist in two forms one of which, in serum, gives a prompt reaction and the other does not. Each of these free acid forms may give rise to salts which behave towards the van den Bergh reagent in the same way as the acids from which they are derived. This explanation assumes that the direct-reacting pigment from bilirubin and that obtained from bile are identical, and that in the preparation of the former we have succeeded in preparing from bilirubin of one form the sodium salt of the other form.

A search through the literature on bilirubin showed that the idea of multiple forms of bilirubin and its salts is not new, for Küster [1915, 1917] arrived at a similar view on totally different grounds. In particular Küster [1917] described the preparation of two forms of ammonium salt, and from these he obtained different forms of free bilirubin. In view of the results which have already been described, it seemed to be of special interest to examine the behaviour of Küster's ammonium salts, when dissolved in serum, towards the van den Bergh reagent. For their preparation bilirubin is suspended in methyl alcohol and dry ammonia gas passed into the liquid. Next, instead of precipitating the ammonium salt at once with ether, as in the earlier method, the solution is placed in a cooling mixture and a portion of the pigment crystallises out. This is Küster's crystalline "Bilirubinammonium." The filtrate from it is then treated with ether and the precipitate produced is the second ammonium salt, Küster's ether-precipitated "Bilirubinammonium." Küster used bilirubin obtained from ox gallstones.

In the present work the crystalline bilirubin from human gallstones was first used. The crystalline ammonium salt prepared from it when added to

serum gave a delayed reaction, while the ether-precipitated salt gave a reaction which was biphasic. A preparation of the ammonium salts was then made from the precipitated bilirubin described in our method for the isolation of bilirubin from gallstones. In this case the quantity of crystalline ammonium salt obtained was extremely small, and it gave a delayed reaction. Fractional precipitation with ether was carried out on the mother-liquor and three fractions of precipitated salt were obtained. All gave a biphasic reaction, but the last fraction gave the reaction which was most rapid and most nearly approached a prompt one.

The above was repeated, using sodium salts prepared by suspending bilirubin in methyl alcohol and adding sodium ethoxide until the bilirubin dissolved. When crystalline bilirubin was used, the crystalline sodium salt gave a delayed reaction; of two separate fractions of ether-precipitated salt, the first gave a delayed reaction while the second gave a biphasic reaction of fair rapidity. With precipitated bilirubin the quantity of crystalline sodium salt obtained was negligible; of two fractions of ether-precipitated salt the first gave a delayed reaction, the second a biphasic one.

These results show that in addition to the differences pointed out by Küster between his two salts of bilirubin, there is also some difference in their behaviour towards the van den Bergh reagent, but the difference is not so sharp as between the two salts we have previously dealt with. This does not entirely disprove an essential identity between the ether-precipitated salt of Küster, which gives biphasic reactions, and the prompt-reacting pigment prepared from bilirubin, or that isolated from bile. In the first place it could hardly be expected that the method of separation used by Küster would give pure products unless carried out under very carefully controlled conditions; the ether-precipitated product probably contains a certain amount of the more slowly reacting form of salt, so that the reaction obtained would not be so rapid as would be the case if complete separation from the crystalline salt had been attained. Further, it is possible that Küster's precipitated salt may be a prompt-reacting pigment when first formed, but that in the absence of stabilising agents, such as alkaline salts or certain constituents of bile, changes occur which result in some loss of direct-reacting properties.

Crystalline and precipitated bilirubin.

In view of the differences which have been noted above between the products obtained from crystalline bilirubin and those obtained from precipitated bilirubin, as well as differences which appear in the statements of different workers with respect to certain properties of the bilirubin they had employed, we were led to make a further examination of these two substances. Difference in colour is certainly evident, but this of itself is of little importance. But there are other differences which are of significance. Thus, the crystalline form is decidedly less soluble in chloroform than the precipitated form. The former is insoluble in serum and in cold sodium bicarbonate solution, while the latter is

soluble in both. These differences are precisely those noted by Küster in what he claimed to be different modifications of bilirubin, isolated respectively from his two ammonium salts, and the two substances we have been dealing with are probably identical with Küster's two modifications. We have further found that the two products differ in their behaviour towards the van den Bergh reagent. If crystalline bilirubin is suspended in serum, the mixture only reacts very slowly with the reagent, whereas a solution of the precipitated form in serum reacts much more rapidly, giving a result which, though not prompt, might in most cases be justifiably described as biphasic.

With regard to the possible identity between the precipitated bilirubin and the prompt-reacting free bilirubin obtained from bile, we have to apply the same considerations as those we have just used in discussing the relationship between Küster's ether-precipitated salt and the bilirubin salt from bile.

The ammonia content of bile.

Küster showed that his ammonium salts lose their ammonia when they are heated with chloroform or methyl alcohol, and also when subjected to a vacuum. In the isolation of direct-reacting pigment from bile, the conditions have been such, therefore, that if the pigment had originally been present as ammonium salt it is probable that it would not appear as such in the final product; the fact that the latter appears to contain a sodium salt may be due to replacement of ammonium by sodium at some stage of the process which has been employed.

Direct-reacting pigments from bilirubin, whether prepared as potassium, sodium or ammonium salts, have so far shown nothing in their general behaviour or in their behaviour towards the van den Bergh reagent to distinguish them from one another, so that there has been nothing in our previous results which definitely disproves the suggestion of Collinson and Fowweather that the direct-reacting pigment may be an ammonium salt.

One of the reasons which caused these workers to make this suggestion was the loss of direct-reacting properties which occurs when sera from cases of obstructive jaundice are heated, or kept. Since, in their view, the difference between the direct-reacting and indirect-reacting pigments was that the one was a salt of bilirubin and the other the corresponding free acid, this loss of direct-reacting properties appeared to indicate a change from salt to free acid, and such a change is most likely to occur if the salt is an ammonium salt.

The present work however, since it proves that indirect-reacting salts exist, as well as direct-reacting salts, shows that it is not necessary to assume that a change to free acid must occur when direct-reacting properties are lost, and that therefore this loss is of no value as evidence in favour of the existence of the direct-reacting pigment as ammonium salt.

Another fact which influenced Collinson and Fowweather was that they found that bile, which contains much more pigment than blood, also contains

much more ammonia, but beyond establishing this fact in a general way by comparative tests, they did not obtain actual figures.

It was thought advisable to return to this point and make quantitative determinations of the ammonia in bile. The method employed was that used by Nash and Benedict [1921] for ammonia in blood, except that the amount of ammonia present allowed of its determination by titration instead of by nesslerisation. Eight determinations were made, seven on human gall-bladder bile obtained from the *post-mortem* room, and one on dog's bile obtained from the animal within half an hour of death. The results are as follows.

Case	Time after death hrs.	Description of bile	Ammonia mg. per 100 cc.
1 (human)	29	Fairly thin, dark brown	30·4
2 ,,	12	Thick, almost black	7·85
3 ,,	13	Thin, dark brown	3·83
4 ,,	3	Fairly thin, golden	2·11
5 ,,	6	Thick, brown	7·66
6 ,,	5	Thin, golden	2·87
7 ,,	34	Fairly thick, dark brown	32·7
8 (dog)	½	Thin, brown	1·03

The ammonia content of human blood is less than 0·3 mg. per 100 cc.

The above series of estimations on bile is small, and is simply intended to give a general impression as to the amount of ammonia in bile compared with that in blood. It is known that the ammonia content of blood increases if the specimen is allowed to stand. The above figures for bile suggest that here too a similar increase may occur, since the highest figures are obtained in the cases in which the greatest interval of time has elapsed between death and analysis of the specimen. Probably only the lower figures in the Table approximate to the actual ammonia content of fresh bile, but considering these alone, there seems to be sufficient evidence that bile contains more ammonia than blood.

The approximate average of the three lowest figures for human bile is 3 mg., and this, if present as ammonium bilirubinate, would correspond to 0·103 g. of bilirubin.

Many of the analyses of bile which are to be found in the literature do not give separate figures for bile pigment, but give a single figure for mucin and pigments. Of twelve such analyses relating to human gall-bladder bile, collected by Rosenbloom [1913], the highest figure for mucin and pigments is 4·44 % (by weight), and the lowest 0·23 %. The mean of the twelve values is 2·23 %. Evans [1930] quotes an analysis by Czyhlarz *et al.* in which mucin and pigments together amount to 1·80 %, mucin being responsible for 1·64 % and pigment for 0·16 %, *i.e.* the figure for pigment constitutes one-eleventh of the total of mucin and pigment. On this basis the analyses quoted by Rosenbloom would give an average value of 0·2 % for pigment, with considerable divergence in individual analyses on either side of this value. The figure for bilirubin, *viz.* 0·103 %, which has been calculated as equivalent to the mean of the three lowest figures for ammonia in the results above, may therefore be reasonably

taken to fall within the range shown by actual analyses of bile, and is quite close to the figure quoted by Evans. This is not taken to be a proof, or even a confirmation, of the view that the natural salt of bilirubin present in bile and showing direct-reacting properties, is the ammonium salt, but it does show that what we know of the ammonia and pigment content of bile is not inconsistent with this view. The possibility therefore that the natural direct-reacting pigment may be an ammonium salt still remains until we have further information to prove or disprove it. Simultaneous determinations of ammonia and bilirubin in bile, or in sera from cases of obstructive jaundice, might reasonably be expected to give further information on this point.

Discussion.

The results just reported establish beyond reasonable doubt the fact that free bilirubin may exist in more than one form, and that corresponding to these different forms are different series of salts, and the recognition of this fact explains the conflicting claims which have been made by different workers as to certain important properties of bilirubin. Newman, for example, states that his bilirubin dissolved in serum and gave a prompt reaction, but his bilirubin was obtained from bile by a process very similar to that by which Griffiths and Kaye obtained their direct-reacting pigment, and it is evident that he was dealing with the direct-reacting, serum-soluble form. Collinson and Fowweather on the other hand have claimed that bilirubin is insoluble in serum and when suspended in serum gives a delayed reaction. Their product however was the crystalline bilirubin obtained from gallstones, and it is thus easy to see why they differed from Newman. In general, much conflicting evidence on the bilirubin problem can be explained when it is realised that where different results are claimed it is due to the fact that different forms of bilirubin have been used.

In the light of the present findings the explanation of the van den Bergh reaction appears to be as follows.

The pigment of sera from non-obstructive cases of jaundice, which only give a delayed reaction, is bilirubin in the more stable, slowly reacting form, which we shall refer to as bilirubin A. Much of the evidence previously obtained suggests that this is in the free acid form, but in view of the fact that its salts are also non-direct-reacting, and soluble in chloroform, the possibility of its being present as a salt requires further consideration and investigation. The pigment in sera from cases of obstructive jaundice, and in bile, is a salt of the less stable, more soluble, prompt-reacting form, which we shall call the B form. Bilirubin B and its salts have a great tendency to change into the A form, and this accounts for the loss of direct-reacting properties observed in old specimens of bile, or in obstructive sera which have been heated or allowed to stand for some time. Whether the salt is the ammonium salt or the sodium salt is not yet clear; this question has already been discussed in dealing with the ammonia content of bile.

From what has been said of the properties and mode of formation of the

direct-reacting salts, it will be evident that if the pigment of natural obstructive jaundice sera is such a salt, states of acidosis will have an unfavourable effect on the formation or the stability of such a pigment. This probably explains why M'Gowan found some relation between the alkali reserve of the blood and the type of reaction. We have also shown that the stability of the direct-reacting pigment, as such, is apparently dependent on other material derived from bile. Hence in slight degrees of obstructive jaundice it is probable that the amount of stabilising substance passing into the blood along with the pigment is often insufficient to maintain the latter in its prompt-reacting form. There will thus be some relation between the concentration of pigment and type of reaction, but we do not agree with Snider and Reinhold that concentration is the only factor concerned. No doubt the general view that the nature of the pigment in icteric sera is dependent on the nature and mechanism of the jaundice is true, but if it is recognised that it is also dependent on such factors as the alkali reserve of the blood, and the concentration of certain stabilising substances, it will be realised that these latter factors may sometimes obscure the original relationship between type of pigment and nature of jaundice. This explains why sera are occasionally met with whose reactions are not in agreement with the nature of the jaundice which is present, and so have led to some disappointment in the application of the van den Bergh reaction as a clinical test and aid to diagnosis.

The explanation offered by Küster to account for the different forms of bilirubin is that in this substance we have an example of keto-enol tautomerism. He considers bilirubin A to be a keto-form, while bilirubin B is an enol modification. He also calls attention to the possibility that between these two forms intermediate products may exist, and this is to be borne in mind as a possible explanation of our failure to establish definitely an identity between certain substances we have dealt with.

Küster offers graphic representation of his views, based on his formula for bilirubin as a tripyrrylmethane compound. The "enol" form is represented in two ways, each showing two hydroxyl groups and the "keto"-forms are also represented by two formulae, each showing one hydroxyl and one keto-group. Küster's formula for bilirubin has been opposed by H. Fischer, who represents bilirubin as a tetrapyrrylethylene [see Fischer and Postowsky, 1926], and assigns to it a formula which allows of different forms as a result of stereo-isomerism as well as indicating the possibility of tautomerism of keto-enol type. Fischer's formula is the one most generally accepted.

Little is known concerning the actual mechanism of the coupling between bilirubin and diazo-compounds. Pröscher [1900] isolated what he believed to be a mono-azo-compound, as a result of coupling bilirubin with diazotised aminoacetophenone. But he assumed a formula $C_{16}H_{18}O_3N_2$ for bilirubin, and if his results are re-calculated on the basis of the modern formula $C_{33}H_{36}O_6N_4$, it is seen that he was really dealing with a bis-azo-compound. Orndorf and Teeple [1905] showed that when bilirubin couples both mono- and

bis-azo-compounds are formed. Fischer and Barrenscheen [1921] confirmed these findings, but did not arrive at any definite conclusion as to the nature of the coupling which occurred. Küster's formula does not appear to indicate how the coupling might take place according to the usual conceptions of the formation of azo-colouring matters, but a modification of Fischer's formula, along the lines suggested by an earlier form given by Fischer and Röse [1914] would provide two unsaturated side-chains CH_2: CH. which form part of a butadiene type of structure. Meyer [1919] showed that typical azo-derivatives could be obtained by coupling suitable diazo-compounds with butadienes. Hence we should have in this modified Fischer's formula an indication of how the coupling might take place, and give mono- or bis-azo products according as one or both side-chains took part in the reaction. The difference in rate of coupling shown by the two forms of bilirubin might be explained on the grounds that the presence of the OH group in the enol form might favour rapid and complete coupling in much the same way as does a phenol group in aromatic compounds.

The fact that both forms of bilirubin couple rapidly when dissolved in alcohol (as in the indirect van den Bergh reaction) is also not inconsistent with the assumption of a keto-enol tautomerism, for there are many cases of such tautomerism in which it has been shown that solution in alcohol favours the change towards the enol modification. It has been noticed in the present work that crystalline bilirubin when dissolved in alcohol oxidises much more rapidly than when dissolved in chloroform, suggesting that in the former solvent a greater amount of the more reactive, more readily oxidisable form is present than in the latter solvent.

The mechanism of the process by which free bilirubin A is converted into salts of bilirubin B remains to be considered. The process requires in the first place solution of the bilirubin in alkali. This would be expected to favour a change from keto- to enol-form. The precipitation of the prompt-reacting form from this solution by the addition of concentrated solutions of alkaline salts might be explained on the grounds that the latter, while offering a sufficiently alkaline reaction to maintain the enol form, also exert a salting out effect on this form, so that it appears as a precipitate. The successive changes are:

Free bilirubin A → salt of A → salt of B.

When the precipitate is dissolved in water, the absence of alkali allows rapid change again towards the keto-modification, so that when the solution is added to serum no prompt reaction is obtained. If the solid itself is added to serum there will also be a tendency to change to keto-form, but the presence of bicarbonate in serum is likely to have a retarding effect on this change; serum-proteins too may have a similar influence, so that we get a prompt reaction for some time, but that finally a change occurs in which most of the pigment passes into the keto-form is seen in the fact that both natural and artificial sera ultimately lose the capacity to give prompt reactions.

Along these lines it appears possible to explain most of the experimental results, but when we consider the formation of the prompt-reacting pigment within the body we meet with certain difficulties. If this is assumed to be the same as the process we have just dealt with, the solution of the free bilirubin A would require a degree of alkalinity which, in view of what we know of the reaction of body fluids and tissues, seems impossibly high, for no appreciable solution of free bilirubin A takes place in the cold below a p_H of 10·5, and at 37° the alkalinity required is only slightly less. The rest of the process, namely change of salts of A to salts of B, is not difficult to understand. We have seen that certain substances in bile appear to have the property of stabilising the B forms. These will be present in the cells of the liver, in which the change from indirect to direct pigment is believed to take place, and it seems probable that besides stabilising B forms (possibly by forming loose combinations with them) they may also have considerable influence in favouring their production. But the whole change might conceivably occur as follows:

Free bilirubin A → free bilirubin B → salts of bilirubin B.

Some tendency towards equilibrium between the two forms of free bilirubin, even in the solid state, is indicated by Küster, who states that if either form is stored as solid for some time a certain amount of the other form begins to appear. In the state in which the indirect pigment exists in the body, changes may be expected to occur more rapidly than in the dry, solid form, and may perhaps be accelerated by those substances in the liver cells which stabilise the B modification when it is formed. Moreover free bilirubin B is soluble in serum. Hence solution and formation of the B salt may, by this process, require no greater alkalinity than that of blood.

But there is another possibility: bilirubin, when formed from haemoglobin, may first appear in the B form. This would dissolve in the blood as a salt of this form, but in the absence of stabilising substances might change rapidly to the A form of salt. Passage through the liver cells would have the effect of reconverting it to the B form and stabilising it as such. On this view, the indirect pigment would be a salt of bilirubin A, and not the free acid—a possibility which has already been referred to.

If this view is correct, we should not expect bilirubin to appear as an ammonium salt. The liver is the only site in which the formation of an ammonium salt is at all likely to occur. If a salt is formed elsewhere it will probably be the sodium salt, and we should not expect the sodium to be subsequently replaced by ammonium, either in the liver or elsewhere. Hence, if it can be shown that the direct-reacting pigment is an ammonium salt, it will follow that the liver is the site in which salt formation first takes place.

The recognition of the fact that bilirubin may exist in more than one form thus raises many questions as to the structural difference between these forms, the mode of change from one form to another and the mechanism of the coupling with diazo-salts. None of these questions can be regarded as settled,

and, while possible explanations have been suggested, the incompleteness of the evidence and the need for further work are fully recognised.

The original object of the present work, however, was to throw some light on the problem of the van den Bergh reaction, and the recognition of the existence of more than one form of bilirubin undoubtedly clears up many of the discrepancies and contradictions in recent work on this subject. In that, we believe, lies sufficient justification for the publication of these results at the present stage.

SUMMARY.

1. Red bilirubin, obtained from gallstones by crystallisation from chloroform solution, is insoluble in serum and gives a delayed van den Bergh reaction when suspended in serum. The pigment obtained by precipitation from chloroform solution after removal of the crystalline bilirubin is soluble in serum and gives a much more prompt reaction than the crystalline pigment.

2. Certain salts which have been prepared from crystalline bilirubin also fail to give a prompt van den Bergh reaction.

3. A pigment isolated from bile after acidification is soluble in serum, in which it gives a prompt reaction. A pigment having all the characteristics of a sodium salt, isolated from bile without acidification, also gives a prompt reaction when dissolved in serum. It is concluded that these pigments are related to one another as free acid and salt.

4. A method has been described by which salts giving a prompt reaction when dissolved in serum may be prepared from crystalline bilirubin.

5. It is concluded from the foregoing that bilirubin may exist in two different forms, each giving rise to a separate series of salts.

6. Attention is called to the work of Küster, who reached similar conclusions along different lines, and the relation between Küster's different forms and those dealt with in this work is considered.

7. An explanation of the van den Bergh reaction is offered in which it is considered that the indirect pigment is bilirubin of one form, but whether as free acid or salt is not yet clear, while the direct pigment is a salt of the other form. Recent work on the van den Bergh reaction has been examined in the light of this view.

8. The ammonia content of bile has been determined and the results examined as to their bearing on the possibility that the direct pigment in bile may be an ammonium salt.

9. Küster's views, which regard the two forms of bilirubin as keto- and enol-modifications, have been stated and considered with reference to the structural formulae for bilirubin proposed by Küster and Fischer respectively.

10. The mechanism of the coupling of bilirubin with diazo-salts has been considered in relation to the theory of the keto-enol tautomerism of bilirubin.

11. The mode of transformation of bilirubin from one form to another *in vitro* and *in vivo* has been discussed.

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XXIII. VARIATIONS OF SERUM-CALCIUM IN THE RABBIT.

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In continuing the investigation of the relation of blood-calcium to the formation of cataract in rabbits, we found that there are certain conditions under which the serum-calcium shows considerable variations. It is true that one of us [Adams, 1930] previously obtained satisfactory control experiments in which only very slight variations were observed; but, with repeated experiments, it became apparent that the level of serum-calcium may vary considerably in individual rabbits, over long periods, and in particular with different diets.

The evidence of other observers is conflicting. Thus Davies, Dickens and Dodds [1926] state that in individual rabbits the serum-calcium does not vary more than 1·5 mg. per 100 cc. serum. Ssewerin [1927] states that the serum-calcium level remains unchanged when cabbage is fed to a starving rabbit. He gives no figures. On the other hand, Ipponsugi [1927, 1], in fifteen control rabbits, found the serum-calcium varying from 13·5 to 18 mg. per 100 cc. and in ten other controls he found variations from 12 to 17 mg. per 100 cc. Harnes [1928] determined the serum-calcium in eighty rabbits throughout one year and found variations from 14·5 to 18·5 mg. per 100 cc. Culhane [1927] reports variations, in six rabbits fed on oats and cabbage, from 14·31 to 17·12 mg. Ca per 100 cc. serum. Kapsinow and Underhill [1929] observed considerable daily variations in the serum-calcium level, usually between 10 and 15 mg. per 100 cc. serum, with one figure as high as 21 mg. per 100 cc.

In addition there exists a further uncertainty as to the effect of diet[1] on the serum-calcium in this animal. Culhane [1927, 1930] reported that cabbage causes a rise in the serum-calcium, but Kapsinow and Underhill [1929] were unable to confirm this. Cannon and Greenwood [1930] found that a diet of sweet clover caused the serum-calcium of young rabbits to decrease.

[1] After this paper had been sent to press, we received the copy of the *Journal of Biological Chemistry* for December, 1931, containing an article by Dupré and Semeonoff [1931] in which the effects of diet on serum-calcium and phosphorus in the rabbit are described. The results obtained by these authors are in agreement with those which we report in this paper.

Because of the importance of the rabbit as an experimental animal, and the uncertainty which seems to exist regarding the normal serum-calcium in this animal, it seemed worth while to attempt to determine more exactly the conditions under which the serum-calcium is variable, and to ascertain how these variations are related to diet.

Experimental.

Our investigations may be summarised as follows.

I. Normal variations.
   (*a*) A general comparison of estimations of serum-calcium from a number of animals over a considerable period.
   (*b*) A comparison of estimations of serum-calcium of animals which had fasted for 16 hours before the taking of the blood sample.

II. A study of the effect of diet on
   (*a*) The serum-calcium of fasting animals.
   (*b*) The reaction and calcium content of the urine.
   (*c*) The calcium and inorganic phosphorus of the serum and urine.

III. Estimations of haemoglobin, as a control on the effect of haemorrhage, whenever an animal was bled repeatedly.

Methods.

The methods used were as follows.

Blood samples. These were taken from the marginal ear vein. The ear was warmed gently, and a small slit made with a flat surgical needle of the Hagedorn type. The size of the slit was regulated to the amount of blood required. The blood flowed quickly and the bleeding was easily stopped when the required amount of blood had been obtained.

Calcium. The Clark and Collip [1925] modification of the Kramer and Tisdall method was used in all cases. The blood was allowed to clot overnight, and after the addition of oxalate the tubes were again allowed to stand overnight to insure complete precipitation. Care was taken that all experimental details were as constant as possible.

Inorganic phosphate in serum. The method of Fiske and Subbarow [1925] was used. The blood for this determination was drawn into a centrifuge-tube. As soon as it had clotted, the clot was loosened from the side of the tube, and the whole centrifuged at high speed for about 10 minutes. 2 cc. of clear serum were used for each determination.

Urinary calcium. This was determined by the method of McCrudden [1911]. If the urine was alkaline, it was made slightly acid to litmus with HCl and filtered. The calcium was precipitated from 10 cc. of the filtered urine, redissolved in sulphuric acid and determined by permanganate titration.

Inorganic phosphate in the urine. This was determined by the Fiske and Subbarow [1925] method, a portion of the slightly acidified filtered urine being used.

Haemoglobin. A Salhi haemoglobinometer was used.

Results.

I. *Normal variations.*

(*a*) *General comparison of estimations from different animals.* We have collected the results of our experience with 18 buck rabbits over a period of about one year. These animals were kept in separate cages and were well housed and cared for. They were given a plentiful supply of oats and a variable amount of cabbage once a day. The figures obtained show considerable and irregular variations in the serum-calcium of individual rabbits over a period of time and also variations among different individuals bled at approximately the same time. These results were obtained on blood samples taken at random from normal animals without reference to diet or to the length of time elapsing after feeding.

The lowest value obtained was 12·4 mg. calcium per 100 cc. serum, and the highest 18·6 mg. per 100 cc. serum. The maximum variation for a single animal was 5·6 mg.

(*b*) *A comparison of estimations from animals which had fasted for 16 hours before the taking of the blood sample.* In order to find the effect of more constant conditions on the serum-calcium of rabbits, we tried withholding food from the animals for 16 hours before taking the blood sample for calcium determination. The routine was that the animals were allowed to feed freely for 8 hours during the daytime; all food was removed from the cages in the evening, and the animals fasted for 16 hours. The blood samples for calcium determination were taken on the following morning, at the end of the fast and before the beginning of the day's feeding. The results are given in Table I. They show that under these conditions the serum-calcium is more nearly constant than in blood samples taken at random from animals presumed to be continuously feeding.

The lowest value obtained in the fasting animals was 12·3 mg. per 100 cc. serum (No. 15, Table I), and the highest, 15·3 mg. per 100 cc. serum (No. 10). The maximum variation among 10 animals under the fasting conditions described was therefore 3 mg. as contrasted with a variation of 6·2 mg. among the feeding animals.

The maximum variation for an individual rabbit under the fasting conditions was 1·6 mg. (No. 18, Table I), whereas among the feeding rabbits, the maximum variation for a single individual was 5·6 mg.

In Table I we have indicated the type of diet fed prior to the 16-hour fasting period. The diet was varied, sometimes oats alone or cabbage alone, or a mixture of oats and cabbage was given. As will be seen from Table I, the type of diet fed prior to the fast does not appear to have any striking effect on the

Table I. *Showing the relative constancy of the fasting blood-calcium level.*

Rabbit No.	Weight g.	Date	Haemoglobin % initial	Serum-calcium mg. per 100 cc.	Diet prior to fasting 16 hrs.
7	2000	5. v. 31	—	15·3	Oats and cabbage
		6. v. 31	—	13·8	Oats and water
		7. v. 31	—	14·2	Oats and water
		9. v. 31	—	13·9	Cabbage
9	1625	28. iv. 31	100	15·2	Oats and cabbage
		29. iv. 31	100	14·9	Oats and water
		30. iv. 31	—	14·4	Oats and water
		27. v. 31	100	13·9	Oats and cabbage
		29. v. 31	—	13·7	Oats and cabbage
		4. vi. 31	94	13·6	Oats and cabbage
10	1875	28. iv. 31	—	15·2	Oats and cabbage
		29. iv. 31	—	15·3	Oats and water
		30. iv. 31	—	13·9	Oats and water
15	1625	14. iv. 31	100	13·8	Oats and cabbage
		15. iv. 31	100	13·0	Cabbage
		16. iv. 31	96	13·7	Oats and water
		17. iv. 31	88	12·3	Cabbage
		26. v. 31	100	13·5	Oats and cabbage
		29. v. 31	—	13·4	Cabbage
		4. vi. 31	113	13·5	Cabbage
16	2125	6. v. 31	—	14·1	Oats and cabbage
		7. v. 31	—	14·6	Oats and water
		8. v. 31	—	13·3	Cabbage
17	1725	14. iv. 31	100	13·9	Oats and cabbage
		15. iv. 31	97	13·5	Cabbage
		16. iv. 31	92	13·7	Oats and water
		17. iv. 31	97	13·6	Cabbage
		13. v. 31	100	14·8	Cabbage
		14. v. 31	—	14·2	Cabbage
		15. v. 31	86	13·5	Cabbage
		18. v. 31	—	14·3	Cabbage
		19. v. 31	80	14·2	Cabbage
		22. v. 31	81	14·6	Oats and water
18	2375	13. v. 31	100	13·9	Oats and water
		14. v. 31	—	14·4	Oats and water
		15. v. 31	83	13·6	Oats and water
		19. v. 31	77	14·5	Oats and water
		21. v. 31	—	15·1	Oats and cabbage
		22. v. 31	70	15·2	Cabbage

fasting blood-calcium level, although in one case (No. 18) there appears to be a definite rise in the fasting level when cabbage is fed after an oats diet of 4 days' duration.

In the experiments on fasting animals, and in all subsequent work where repeated determinations were made on the same animal, haemoglobin estimations were included as a check on the effect of bleeding. The evidence in the literature as to the possible effect of blood loss on the serum-calcium is conflicting. Kapsinow and Underhill [1929] found a fall in the haemoglobin content as a result of repeated withdrawal of blood samples for calcium determination, and suggested that this might materially affect the serum-calcium value. Clark [1920] showed that extensive haemorrhage caused a fall in the serum-

calcium of rabbits, whereas Stewart and Percival [1927] found that a withdrawal of 30 to 48 cc. of blood, causing a 20 to 42 % reduction in the haemoglobin content, caused an 8·6 to 20 % reduction in the serum-calcium. Adams [1930] obtained a variation of not more than 1 mg. in four successive estimations in 24 hours, in any one rabbit, 6 to 8 cc. being taken for each sample.

In this work we have taken the precaution to avoid possible complicating factors due to haemorrhage by avoiding too frequent bleeding of the same animal, and in addition we have included control estimations of the haemoglobin content of the animal's blood.

II. *The effect of diet.*

The fact that the serum-calcium was found to be higher and more variable in the feeding than in the fasting animals, together with the statement by Culhane [1927, 1930] that cabbage causes a rise in the serum-calcium of rabbits, led us to study systematically the effect of diet on serum-calcium, in the hope of finding an explanation of the extraordinary variability of the blood-calcium level in the rabbit.

(*a*) *The effect of a meal of oats and of cabbage on the serum-calcium of fasting animals.* Table II shows the effect of cabbage and of oats on the serum-

Table II. *Showing the effect of cabbage and of oats on the serum-calcium of fasting rabbits.*

Rabbit No.	Date	Serum-calcium, mg. per 100 cc. Fasting	2–2½ hrs. after food
	Cabbage.		
7	7. v. 31	14·2	16·8
9	27. iv. 31	14·4	16·4
10	27. iv. 31	13·9	16·1
14	9. iv. 31	13·3	15·9
14	10. iv. 31	11·3	15·9
15	10. iv. 31	13·9	15·2
15	16. iv. 31	13·7	14·8
16	10. iv. 31	12·5	13·9
17	13. iv. 31	13·9	15·2
17	16. iv. 31	13·9	14·3
18	25. iv. 31	14·7	16·8
	Oats.		
7	4. v. 31	15·3	14·7
9	27. iv. 31	15·2	15·1
10	28. iv. 31	15·2	14·4
16	5. v. 31	14·1	14·2
16	8. v. 31	13·9	12·4

calcium of fasting animals. In each case the first determination was made after a fast of 16 hours, and the second in 2 to 2½ hours after a liberal meal of cabbage or of oats. In order to compensate for the dryness of the oats, animals on this diet were allowed access to water.

In all the eleven experiments with cabbage there was a rise in the serum-calcium 2 hours after feeding. The maximum rise observed was 4·6 mg. in rabbit

No. 14 (Table II); the minimum, 0·4 mg., in rabbit No. 17. The average rise 2 hours after cabbage was 1·8 mg.

Of the five animals to which oats were given instead of cabbage, four showed a fall in serum-calcium 2 hours after feeding, while one showed a rise of 0·1 mg. The maximum fall was 1·5 mg. and the minimum, 0·1 mg. The average fall on the oats diet was 0·62 mg. as contrasted with an average rise on the cabbage diet of 1·8 mg.

(*b*) *The effect of diet on the reaction and calcium content of the urine.* The animals were placed in small metabolism cages, and the urine was collected separately from the faeces. On a diet of cabbage only, a large volume of strongly alkaline urine was excreted. This urine was of a pale yellow colour, and contained varying amounts of white precipitate. The precipitate went into solution with the evolution of much CO_2 when the urine was acidified with HCl. Such urine invariably contained large amounts of calcium, probably excreted in solution as calcium bicarbonate, but precipitating out as calcium carbonate when CO_2 was lost as a result of allowing the urine to stand exposed to the air.

On a diet of oats alone, a small volume of acid urine was excreted. The urine varied in colour from pale straw to dark brown, and almost always contained a quantity of gelatinous material. The calcium content of the urine was much less on the oats than on the cabbage diet.

Typical results from two animals are given in Table III. The total volume of urine excreted by rabbit No. 18 on a cabbage diet in 24 hours varied from 275 to 632 cc. In rabbit No. 17, on an oats diet, the volume varied between

Table III. *Effect of diet on the reaction and calcium content of the urine.*

Rabbit No.	Date	Total calcium mg. in 24 hrs.	Volume cc. in 24 hrs.	Reaction to litmus	Food eaten during 24 hrs. g.
18	13. v. 31	279	286	Alkaline	400 Cabbage
	14. v. 31	437	275	,,	500 ,,
	15. v. 31	Lost	486	,,	500 ,,
	16. v. 31	1000	535	,,	500 ,,
	17. v. 31	788	632	,,	500 ,,
	18. v. 31	1060	420	,,	500 ,,
	19. v. 31	884	320	,,	520 ,,
	20. v. 31	985	564	,,	600 ,,
	21. v. 31	905	324	,,	400 Cabbage and 145 Oats
	22. v. 31	125	81	Sl. alk.	58 ,,
17	13. v. 31	54	51	Acid	47 Oats and water
	14. v. 31	32	—	,,	58 ,,
	15. v. 31	38	56	,,	74 ,,
	16. v. 31	40	53	,,	62 ,,
	17. v. 31	7	10	,,	101 ,,
	18. v. 31	32	40	,,	118 ,,
	19. v. 31	37	25	,,	44 ,,
	20. v. 31	0	0	,,	89 ,,
	21. v. 31	169	102	Sl. acid	22 Oats and 200 Cabbage
	22. v. 31	795	292	Alkaline	400 ,,

0 and 57 cc. The animals did not thrive on the diet of oats alone, even when free access to water was allowed. They soon showed loss of appetite, and in some cases developed complete anuria.

The total calcium excretion varied, on the cabbage diet, from 279 to 1060 mg. in 24 hours. On the oats diet the calcium excretion was between 0 and 54 mg. in 24 hours.

(c) *The effect of diet on the calcium and phosphorus content of serum and urine.* As an extension of the foregoing observations, the effects of diet on the serum and the urine were studied simultaneously. As the metabolism of calcium is now known to be related to that of phosphorus in some fundamental way, determinations of the inorganic phosphorus of serum and urine were made in addition to the determinations of serum- and urine-calcium.

The animals were placed in small metabolism cages. They were kept supplied with oats or with cabbage and allowed to feed at will. Six animals were used, and these were divided into two groups. (1) Three were kept on a diet of cabbage only for a period of 6 days, and were then given oats only for a period of 7 or 9 days. (2) The other three were given oats only during the first period and cabbage only during the second period.

The urine was collected separately from the faeces, and the total excretion of calcium and of inorganic phosphorus was estimated from the 24-hour sample.

The calcium and inorganic phosphorus of serum were estimated in blood samples withdrawn at the same time, and these were obtained at any convenient time during the day.

Determinations of calcium and phosphorus in the urine were made every day. Determinations of serum-calcium and phosphorus were not made daily but at intervals in order to avoid too frequent bleeding of the animal. Control estimations of haemoglobin were made in all cases; variations in the haemoglobin content of more than 10 % of the initial value were not found, except in one case (rabbit No. 20, Fig. 3), when there was a fall to about 44 % of the initial value.

The space at our disposal does not permit us to present the results both graphically and in the form of a complete table. We have therefore summarised the results briefly below, and in addition we have selected four experiments, representative of all types of results obtained, which are given in detail in the form of diagrams (Figs. 1 to 4). The diagrammatic form emphasises the interesting relationship between calcium and phosphorus which exists both in the serum and in the urine.

The results of all six experiments may be summarised as follows:

Cabbage diet.

		Maximum	Minimum
Serum	Calcium (mg./100 cc.)	19·3	14·3
	Phosphorus (mg./100 cc.)	4·3	1·5
Urine (alkaline)	Calcium (total in 24 hrs.)	2200 mg.	342 mg.
	Phosphorus (total in 24 hrs.)	Trace	Nil

Oats diet.

		Maximum	Minimum
Serum	Calcium (mg./100 cc.)	15·8	11·0
	Phosphorus (mg./100 cc.)	8·8	3·1
Urine (acid)	Calcium (total in 24 hrs.)	654 mg.	0 (anuria)
	Phosphorus (total in 24 hrs.)	225 mg.	0 (anuria)

Charts showing the effect of diet on calcium and inorganic phosphorus of serum and urine.

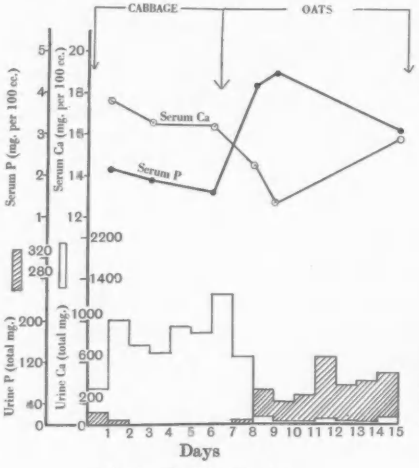

Fig. 1. (Rabbit No. 9.)

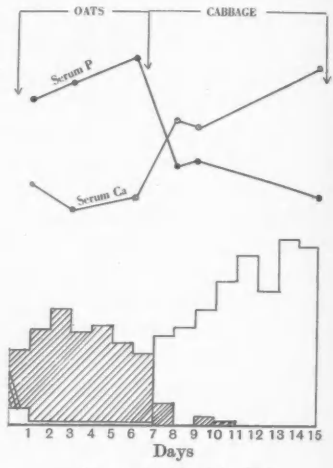

Fig. 2. (Rabbit No. 19.)

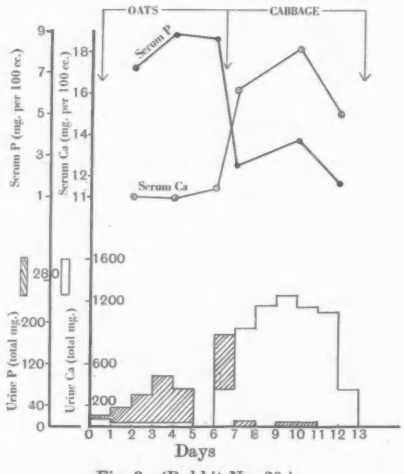

Fig. 3. (Rabbit No. 20.)

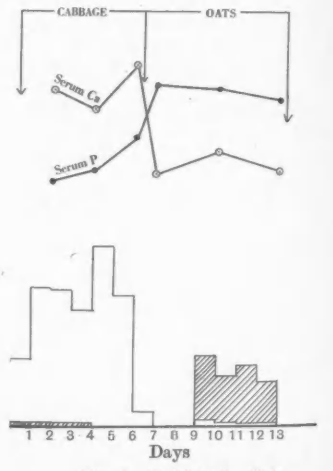

Fig. 4. (Rabbit No. 21.)

In those animals to which cabbage was fed during the first period (see Figs. 1 and 4), there was a rise in the serum-calcium within the first 24 hours from a fasting level (13–14 mg. per 100 cc.) to 16 or 17 mg. At the same time the serum-phosphorus fell. Throughout the period of cabbage feeding, the serum-calcium, although variable, was maintained at a high level. The serum-phosphorus, although also variable, was maintained at a low level and showed a tendency to fall as the serum-calcium rose. The excretion of phosphorus in the urine fell rapidly on the cabbage diet, until, after 2 or 3 days of cabbage feeding, none or only a trace could be detected in the urine. The excretion of calcium on the cabbage diet was variable, but consistently great.

When the diet was changed from cabbage to oats, the serum-calcium rapidly fell and at the same time the serum-phosphorus rose. Throughout the period of oats feeding, the serum-calcium remained at a low level, while the serum-phosphorus was raised; both calcium and phosphorus varied considerably. The urinary excretion of phosphorus was greatly increased on the oats diet, while the excretion of calcium was very much diminished.

In those animals which were given oats during the first period and cabbage during the second (see Figs. 2 and 3), the same effects of diet were observed.

The effect of a change of diet manifested itself more quickly in the serum than in the urine. In shifting from the cabbage to the oats diet, the serum-calcium fell within 24 hours, but there was a lag period of 1 or 2 days during which the urinary excretion of calcium gradually fell and the urinary excretion of phosphorus gradually rose. Conversely, in changing from the oats to the cabbage diet, the phosphorus excretion fell off gradually during 1 or 2 days, while the calcium excretion gradually rose.

It is interesting to note that in the case of both diets the calcium and phosphorus are being influenced in exactly opposite directions. But, whereas the cabbage causes the calcium to increase and the phosphorus to diminish in serum and in urine, the oats diet has exactly the reverse effect.

The fact that diet can affect the reaction of the urine has been known since Claude Bernard [1846] demonstrated that the clear, acid urine of carnivorous animals, and the turbid urine of herbivorous animals result from the character of their diet and not from some fundamental difference in their mode of assimilation and digestion. But only a few detailed studies of the variable effects of different kinds of diet in herbivora are to be found in the literature. In connection with our results, the following observations are of interest.

Warth and Ayyar [1930] observed that cattle excreted an alkaline urine containing large amounts of CO_2 when they were fed on green fodders. Underhill and Bogert [1916] found that rabbits on a diet of oats and corn excreted an acid urine of high phosphorus content. Ipponsugi [1927, 2] found that the excretion of calcium in the urine of the rabbit is variable, and that it is dependent upon the calcium content of the food eaten. He was unable to find any relation between the calcium excretion in the urine and the serum-calcium.

In the experiments of Harnes [1928], who studied the variations in serum-calcium and inorganic phosphorus in rabbits during 8 months, the inorganic phosphorus fell as the serum-calcium increased and *vice versa*.

Discussion.

The chief result of this work is a demonstration of the importance of dietary factors in determining the serum-calcium level in rabbits. It has been shown that the ingestion of cabbage by a fasting rabbit may cause a definite rise in the serum-calcium. In fasting rabbits, and in rabbits feeding upon oats, the serum-calcium was found to lie between 11 and 15·8 mg. per 100 cc., while on a diet of cabbage, values between 14 and 19 mg. per 100 cc. were obtained.

Variations in serum-calcium, such as we have recorded (Section I(*a*)) will probably be observed whenever the blood samples for calcium determination are taken at random, without reference to diet or to feeding-time. In these experiments the animals were fed on oats, and were given a variable amount (50–500 g.) of cabbage once a day. The blood samples were collected either before, or at some unknown interval after, the cabbage meal, with the result that wide variations in the calcium level occur.

The more constant values previously obtained by one of us [Adams, 1930] must be ascribed to fortuitous experimental conditions; possibly chiefly to the coincidence that blood samples were never taken after a heavy cabbage meal.

The effect of cabbage on serum-calcium in rabbits was first noted by Culhane [1927, 1930], who reported that cabbage causes a transitory rise in serum-calcium provided the initial value is not already high. Kapsinow and Underhill [1929] interpreted the findings of Culhane as suggesting that cabbage contains a calcium-raising substance analogous to that found in the parathyroid gland. They attempted to confirm her observation, but, although their experiments gave some indication that cabbage may cause a rise in serum-calcium, this was not sufficient to enable them to conclude that cabbage has the same effect as the parathyroid hormone on serum-calcium. These authors observed considerable daily variations in the serum-calcium level, and they considered that the daily withdrawal of the 5 cc. of blood necessary for calcium determination may possibly interfere with a consistent effect produced by cabbage.

Our results confirm the observation of Culhane [1930] as to the calcium-raising effect of cabbage. But we do not think that this necessarily means that cabbage contains a substance analogous to the parathyroid hormone. It is probable that the raised blood-calcium is the result of an increased absorption of calcium from the intestine. The very great increase in the urinary excretion of calcium, which we have observed on a cabbage diet, suggests that large amounts of calcium are being absorbed.

It is true that in man it is very difficult to alter the blood-calcium level by feeding of calcium salts or foods rich in calcium, but there is evidence to show that in the rabbit this may be more easily accomplished. For example,

McGowan, Cunningham and Auchinachie [1931] found a raised blood-calcium and an increased urinary excretion of calcium, together with a diminished urinary excretion of phosphorus, when 8 % $CaCO_3$ was added to the stock ration given to a rabbit.

The absorption of calcium seems to depend upon the relative amount of phosphorus present, among other factors. When the diet is high in calcium and low in phosphorus, the phosphates are precipitated in the intestine and excreted by the bowel as insoluble calcium phosphate, thus creating a phosphorus deficiency [Peters and Van Slyke, 1931, p. 1107]. The excess calcium is absorbed into the blood to be later excreted by the kidney in some soluble form, such as bicarbonate [Cushny, 1928].

An analysis of the available data on the calcium and phosphorus contents of cabbage and of oats indicates that the Ca/P ratio in these two foodstuffs is widely different.

	Calcium %		Phosphorus %		Ca/P ratio	
Source of data	Cabbage	Oats	Cabbage	Oats	Cabbage	Oats
Our own analyses	0·161*	0·077	—	—	—	—
Sherman [1927]	0·045†	0·069‡	0·029†	0·392‡	1·55	0·17
Kestner and Knipping [1926]	0·200	0·100§	0·085‖	0·401§‖	2·35	0·25
Gregory [1928]	0·051¶	—	—	—	—	—
$Ca_3(PO_4)_2$	—	—	—	—	1·93	

* Outside leaves, such as were given to rabbits.
† Edible portion. ‡ Oatmeal. § Oatflakes.
‖ Given as PO_4, recalculated by us as P.
¶ Average for various New Zealand cabbages.

It appears from these figures that cabbage is relatively rich in calcium, while oats are relatively rich in phosphorus, and this may be the basis of our observations. On a cabbage diet the phosphate present might be precipitated and excreted by the bowel as $Ca_3(PO_4)_2$, causing the phosphate of blood and urine to be markedly diminished. The excess of calcium which the cabbage appears to contain might be absorbed into the blood-stream and eventually excreted in the urine as bicarbonate. Under these circumstances, the regulation of the blood-calcium level would depend upon the rate of excretion of calcium by the kidney, and, in cases of rapid absorption, a transitorily raised blood-calcium might be expected to occur.

The oats diet, on the other hand, with its relatively high phosphorus content, favours the excretion of calcium by the bowel. The absorption of phosphorus and its subsequent excretion in the urine would be increased.

We are inclined to take the view that dietary factors, particularly the ratio of calcium to phosphorus in the diet, may be of great importance in determining the upper limit of the blood-calcium level. But before a hypothesis can be formulated more complete data, such as could be obtained from metabolism experiments, are required.

SUMMARY.

1. The serum-calcium of rabbits may be very variable. Values from 12·4 to 18·6 were found in 18 buck rabbits bled at various times over a period of about one year.

2. If the animals were starved for 16 hours before the taking of the blood sample, the range of variation was narrower, being between 12·3 and 15·3 mg. per 100 cc. serum.

3. The serum-calcium level was found to depend upon the diet.

(*a*) Two hours after the giving of cabbage to fasting rabbits a maximal rise of 4·6 mg., and an average rise of 1·8 mg. per 100 cc., occurred in the serum-calcium. Oats, given to fasting rabbits, caused a maximal rise of 0·1 mg. and an average fall of 0·62 mg. per 100 cc. serum.

(*b*) During continuous feeding on cabbage alone, values between 14·3 and 19·3 mg. calcium per 100 cc. serum were observed. During continuous feeding on oats, the serum-calcium values were between 11 and 15·8 mg. per 100 cc.

4. Simultaneous determinations of calcium and inorganic phosphorus in serum showed that the concentrations of these two substances bear a reciprocal relation to each other. On a diet of cabbage, the serum-calcium increased, while the inorganic phosphorus of the serum decreased. Conversely, on oats a fall in the serum-calcium was accompanied by a rise in the inorganic phosphorus of the serum.

5. The urine excreted by rabbits on a cabbage diet was strongly alkaline to litmus, and contained large amounts of calcium, but no phosphorus, or only a trace.

6. On an oats diet, an acid urine rich in phosphorus and containing relatively little calcium was excreted.

7. The volume of urine excreted in 24 hours on a cabbage diet was more than 10 times as great as that excreted in the same time on a diet of oats and water.

8. It is suggested that dietary factors, such as the calcium/phosphorus ratio in the diet, may be of great importance in determining the blood-calcium level in rabbits.

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XXIV. THE DETERMINATION OF VITAMIN D.

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Dyer [1931] has recently described a method of estimation of vitamin D based on the line test. He took 15 litters each consisting of 7 young rats and treated them as follows. Each litter was fed on a rachitogenic diet for 3 to $3\frac{1}{2}$ weeks, and the different rats in the litter were then given different doses of a sample of irradiated ergosterol obtained from the National Institute for Medical Research. In each litter 1 rat received no irradiated ergosterol and the others received daily doses of $\frac{1}{16}$, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$, 1 and 2 units vitamin D respectively. Dosage was continued for 10 days and then the ulnae and radii of the rats were examined by the line test. Dyer found that a given dose would produce different amounts of healing in different rats. However, by considering all 15 litters he was able to estimate the average healing produced by each dose and to express it in arbitrary figures. He then obtained a smooth curve relating average healing to dose of irradiated ergosterol given.

In similar tests Bills *et al.* [1931] estimated healing by giving one to four + signs to each animal. They examined a large number of rats and found the average healing produced by a given dose and by known multiples of that dose. From these results a curve was obtained relating potency of a solution to the average degree of healing produced.

Two experiments which involved the giving of graded doses of vitamin D to each of several litters of rats have recently been carried out in this laboratory. The results have been calculated in the same way as in Dyer's experiment and have been found to give curves relating the average degree of healing to the dose of vitamin D given. The curves are very similar to Dyer's curves and thus afford additional evidence of the validity of his method of estimating vitamin D.

Experimental.

1. *First experiment.* Ten litters of rats were used. One animal in each litter was given no vitamin D, 4 other animals were given daily doses of vitamin D of 0·25, 0·5, 1·0 and 2·0 units respectively. (The remainder of the animals in each litter were given special diets as described later in this paper.) The ulnae and radii were examined by the line tests and the degree of rickets shown by

each rat was estimated by two independent observers using the arbitrary scale proposed by Dyer. The values assigned to any one animal did not differ by more than 0·5 degree of healing. When this difference occurred, the average of the two values was determined. The results are given in Table I.

Table I.

Litter	Average value for degree of healing shown by rats receiving daily doses of vitamin D: No dose	0·25 unit	0·5 unit	1 unit	2 units
	"Slight" rickets group.				
563	4	6	5·75	6	6
573	4·5	6	6	6	6
653	4	3	3·5	4·75	6
688	3	5·5	5·5	5	6
Average	3·9	5·1	5·2	5·4	6·0
	"Severe" rickets group.				
583	0	3·25	4	6	6
589	0	3	5	4	4·75
620	0·5	4·5	5	5	6
622	0	4·5	4·25	5·5	6
644	0	5·5	4	4·25	6
700	0	2	4	5	5
Average	0·08	3·8	4·4	5·0	5·6

As in Dyer's experiment, the degree of rickets developed by each litter was judged to be "slight" or "severe". When healing was marked by a line, the width of the metaphyses could be seen in all the members of the litter, and the degree of rickets could therefore be judged by examination of all the rats of the litter. Thus the classification of the litter as having "slight" or "severe" rickets did not depend on the results given by the one or two animals which received no vitamin D. When the members of a litter only showed differences in the width of the metaphyses and no "lines" of healing, the grouping into "slight" or "severe" rickets was dependent wholly upon the value given by the rats which received no vitamin D.

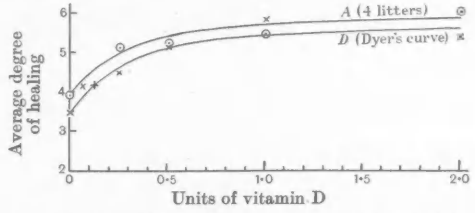

Fig. 1. The relation between dose of vitamin D and the average degree of healing produced in groups of rats which had developed slight rickets.

It will be seen that the animals which received no vitamin D in the 4 litters 563, 573, 653, 688, developed a slight degree of rickets estimated as 3 or more

on the scale of healing, whereas those of the remaining 6 litters developed severe rickets. For each group, the dose of vitamin D was plotted against the average degree of healing produced by the dose. The "slight" rickets group gave curve *A*, Fig. 1, and the "severe" rickets group gave curve *B*, Fig. 2. Curves *D* and D_1 respectively are the corresponding curves given by Dyer. It is evident that these curves and Dyer's curves are very similar.

2. *Second experiment.* Litters of 8 or 9 individuals were chosen for this experiment. In each litter one or two animals were given no vitamin D and at least one animal received one of the following doses of vitamin D daily, 0·0625 unit, 0·125 unit, 0·25 unit, 0·5 unit, 1 unit, 2 units, 4 units. The results were estimated as before and the average values for the degree of healing are shown in Table II. As all these litters developed a severe degree of rickets, they were

Table II.

	Average value for degree of healing shown by rats receiving daily doses of vitamin D							
Litter	No dose	0·0625 unit	0·125 unit	0·25 unit	0·5 unit	1 unit	2 units	4 units
726	0·5	0	4·0	4·7	4·7	5·0	6·0	6·0
741	*0·5	2·8	2·3	4·3	3·5	3·5	4·3	6·0
737	0	1·2	1·2	4·2	4·2	4·8	6·0	*5·5
819	*0·6	0·7	1·8	1·8	2·5	4·2	4·8	5·2
833	0	0·7	1·0	2·2	3·8	4·3	5·2	6·0
818	0·2	0·8	1·3	1·7	3·2	4·5	3·2	4·8
Average	0·30	1·0	1·9	3·15	3·65	4·4	4·9	5·6

* Average of values from 2 rats.

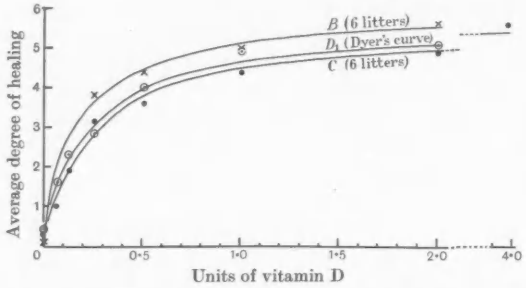

Fig. 2. The relation between dose of vitamin D and the average degree of healing produced in groups of rats which had developed severe rickets.

averaged in one series only. The relation between the dose of vitamin D and the average degree of healing produced in the rats used in this experiment is shown in curve *C*, Fig. 2. Each point on this curve is given by the average of the values obtained from at least 6 individuals. Where 2 rats in a litter received the same dose, the two results were averaged to prevent overweighting of that litter in the average for the dose.

The curve for these litters is also similar to the curve obtained in the first

experiment and to Dyer's curves. As, however, they are not exactly alike, some uncertainty might be felt as to which curve should be used for comparison of two results in the way suggested by Dyer. An attempt has, therefore, been made to see how far a comparison is influenced by the choice of the curve of reference.

The use of different curves for the estimation of the vitamin D potency of an unknown substance.

In the method described by Dyer, the potency of an unknown substance may be compared with that of the standard solution by using either curve D or D_1, according to whether the degree of rickets shown by the animals receiving no vitamin D is less or more severe. The comparison was made by determining the average degree of healing for a given dose of unknown and for a given dose of standard vitamin D. The ratio of the corresponding abscissae on the appropriate curve gave the ratio of the vitamin D contents of the two doses.

Dyer gave an example in which the average value for the degree of healing shown by the animals receiving no dose in 3 litters was 2·8. The values for daily doses of 0·5 g. unknown sample X and 0·5 unit vitamin D were 4·7 and 5·1 respectively. Since these litters developed only moderately severe rickets, the abscissae corresponding to 4·7 and 5·1 degrees of healing were determined by reference to curve D. Hence

$$\frac{\text{Vitamin D content of 0·5 g. sample } X}{\text{0·5 unit vitamin D}} = \frac{0{\cdot}31}{0{\cdot}525} = 0{\cdot}59.$$

Curve A was compiled by a similar method and under similar conditions to curve D. It is not coincident with curve D, but almost parallel to it. This may be due to some unrecognised factor acting in one experiment and not in the other. Suppose the activity of sample X were determined by reference to curve A, then

$$\frac{\text{Vitamin D content of 0·5 g. sample } X}{\text{0·5 unit vitamin D}} = \frac{0{\cdot}17}{0{\cdot}32} = 0{\cdot}53.$$

Thus the potency of sample X would be given as 0·59 unit per g. using curve D and 0·53 unit per g. using curve A. The difference is only 12 % and this in spite of the fact that curve A was constructed from 4 litters only.

Table III. *Showing the use of different curves for determining the potency of an oil when 5 mg. oil gives 3·8 degrees of healing and 0·5 unit vitamin D gives 4·55 degrees of healing.*

Curve	$\frac{\text{Vitamin D content of 5 mg. oil}}{\text{0·5 unit vitamin D}}$	Potency of oil in units per g.
B	$\frac{0{\cdot}28}{0{\cdot}54} = 0{\cdot}52$	52
C	$\frac{0{\cdot}50}{1{\cdot}03} = 0{\cdot}49$	49
D	$\frac{0{\cdot}45}{0{\cdot}87} = 0{\cdot}52$	52

The curves *B*, *C* and D_1 obtained from litters which developed severe rickets may be similarly compared. For example, in a test of a sample of cod-liver oil, the average degree of healing shown by the animals which received no dose was 0·8. The values for 5 mg. cod-liver oil and 0·5 unit vitamin D were 3·8 and 4·55 respectively. The results given by the three curves are shown in Table III. The difference is only 6 %.

It will be seen that the same result is obtained from all three curves. This may be confirmed by taking pairs of imaginary results and evaluating them from each curve.

Discussion.

The division into "slight" and "severe" rickets is necessarily arbitrary. Dyer included in the "severe" group only litters in which the animals receiving no vitamin D gave the value 1·5 or less. Presumably any litter in which the value was greater than this should be included in the "slight" group. In the compilation of curve *D*, however, the lowest value obtained for any rat receiving no dose was 2·6. We have found in practice that when the average value given by the animals receiving no vitamin D in a litter lies between 1·5 and 2·5, the corresponding value for the unknown or for a dose of 0·5 unit vitamin D is often below 3·4. According to Dyer's method, the curve for "severe" rickets is the only one available for the interpretation of such results, since values below 3·4 cannot be measured on curve *D*. All Dyer's results and the results from the experiment here described, were, therefore, collected together to form three curves representing the effect of different doses of vitamin D on litters which developed "slight," "moderate" and "severe" rickets respectively.

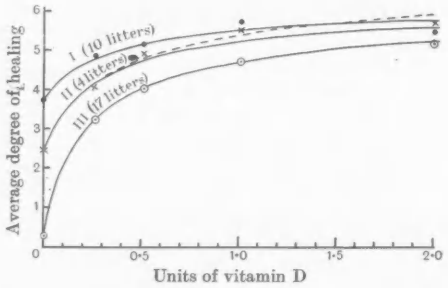

Fig. 3. Summary of all results described in this paper (including Dyer's results).

In Fig. 3, curve I represents the "slight" rickets group in which the value for the degree of healing shown by each animal receiving no vitamin D was greater than 3·0. Curve II represents the "moderate" rickets group in which the values for these animals ranged from 1·0 to 3·0 inclusive. Curve III represents the "severe" rickets group in which the values for these animals lie

below 1·0. For the compilation of these curves the average degree of healing was found for each dose represented in every litter in the group. Curve I was obtained from 10 litters, curve II from 4 litters and curve III from 17 litters. Curve II would be expected to lie between curves I and III as indicated by the continuous line in the diagram. The best curve through the experimental points is, however, that given by the dotted line. This difference is probably due to the fact that only 4 litters were used for the construction of curve II. All the curves are approximately logarithmic in shape.

Comparison of this method with a prophylactic method (ash content of bones) for testing for vitamin D.

A sample of fish meal was examined by the curative method described in this paper and also by a prophylactic method based on the percentage ash in the dry fat-free bones. Details of the two experiments are given below.

1. *Curative method.* The line test was carried out and the results evaluated in the usual way. Three litters of rats were used consisting of 3, 5 and 6 individuals respectively. They all developed severe rickets, the average degree of healing given by the animals which received no dose being 0·7. The average values for the rats which received 2·5 % fish meal in the diet and 0·25 unit vitamin D daily were 5·1 and 4·35 respectively. By reference to curve III (Fig. 3), the ratio $\frac{5·1}{4·35}$ for healing was found to correspond to the ratio $\frac{1·7}{0·7}$ for dose of vitamin D. Records of the total amount of food eaten daily by each rat receiving 2·5 % fish meal, showed that the average amount of fish meal eaten per rat per day was 0·22 g. Therefore

$$\frac{\text{Vitamin D content of 0·22 g. fish meal}}{\text{0·25 unit vitamin D}} = \frac{1·7}{0·7} = 2·43,$$

1 g. fish meal, therefore, contains $\frac{2·43 \times 0·25}{0·22} = 2·75$ units vitamin D.

2. *Prophylactic method (ash content).* This experiment was based on the method described by Chick *et al.* [1926]. In each of the 5 litters used for the test some of the rats received no dose, others received 0·1 unit vitamin D daily and others received 0·5 % fish meal incorporated in their food. The basal diet for all the animals was Steenbock's rachitogenic diet as used in the curative test. The feeding was continued for 28 days, after which the animals were killed and their humeri and femora were removed and dried. The fat was then extracted from the bones with hot alcohol. The percentage ash in the dry fat-free bones was determined for each rat. The results are given in Table IV. The average values for the rats receiving 0·5 % fish meal and 0·1 unit vitamin D daily were 37·3 and 40·1 respectively. These values are not very different from each other but both are higher than the corresponding figure of 29·4 found for the rats which received no dose. The average amount of fish meal eaten per rat per day was 0·039 g. The results show, therefore, that 0·039 g. fish meal contains approximately 0·1 unit vitamin D. 1 g. fish meal,

Table IV.

Showing percentage ash in dry fat-free bones of rats receiving

	No dose		0·5 % fish meal		0·1 unit vitamin D	
Litter	Rat	% Ash	Rat	% Ash	Rat	% Ash
1199	6975	27·0	6977	41·1	6979	46·1
	6976	26·25	6978	37·3	6980	40·7
1193	6940	29·1	6942	32·3	6944	39·4
	6941	33·8	6943	40·5	6945	40·6
1208	7027	31·6	7029	38·0	7031	31·9
	7028	30·1	7030	40·7	7032	40·9
1213	7051	30·2	7052	37·3	7053	42·3
1218	7074	26·8	7075	31·0	7076	39·3
Average values		29·4		37·3		40·1

therefore, contains 2·5 units. Thus the result obtained by this method agrees closely with the value 2·75 units per g. given by the line test.

Since we have no satisfactory curve relating the percentage ash to dose of vitamin D, we cannot evaluate the results given by the prophylactic test unless the dose of unknown substance produces an effect similar to that of the dose of standard as in the example given above. Otherwise the test will only show whether a given dose is more potent or less potent than the dose of standard without indicating how much more, or less, potent it may be. The use of the curve for the interpretation of values, therefore, gives more precise results.

The effect of altering the ratio of calcium to phosphorus in the basal diet.

In the routine testing of the vitamin D content of foodstuffs, it has sometimes been found necessary to incorporate in the diet large quantities of the substance to be tested. It seemed possible that such substances might alter the ratio of calcium to phosphorus in the diet sufficiently to affect the degree of rickets produced in the rats. The rachitogenic property of Steenbock's diet is due to its high value for Ca/P together with its lack of vitamin D. Karelitz and Shohl [1927] showed that when they added to their diet sufficient sodium phosphate to change the ratio of calcium to phosphorus from 4·25 : 1 to 1 : 1 "definite healing" was brought about in 24 to 48 hours. In our routine tests the alteration in the relative amounts of calcium and phosphorus has always been less than this and an experiment was devised to find the effect of small changes in the ratio.

A representative sample of the rachitogenic diet in general use in these laboratories was ground to a fine homogeneous powder in a ball mill. Analysis showed it to contain 1·37 % calcium and 0·341 % phosphorus. The ratio of calcium to phosphorus was, therefore 4 : 1. This diet is referred to in this paper as "diet I." Diets II, III and IV were made with the ratio of calcium to phosphorus 4 : 2, 4 : 3 and 4 : 4 respectively. The ratio was adjusted by the addition of calcium phosphate with the removal of the necessary amount of calcium carbonate. The composition of the diets is given in Table V.

Table V. *Percentage composition of diets.*

	I* Ca : P = 4 : 1	II Ca : P = 4 : 2	III Ca : P = 4 : 3	IV Ca : P = 4 : 4
Yellow maize	76	76	76	76
Gluten flour	20	20	20	20
Sodium chloride	1	1	1	1
Calcium carbonate	3	2	0·85	—
Calcium phosphate	—	1·5	3·0	4·1

* Steenbock's rachitogenic diet No. 2965.

Ten litters of rats were prepared for the test in the usual way by being fed for 3 to 3½ weeks on the rachitogenic diet (No. I) in which the ratio of calcium to phosphorus was 4 : 1. In each litter one or two rats continued to receive this diet during the 10 days' test, 3 other rats received diets II, III and IV respectively, and the remaining animals received diet I together with daily doses of 0·25, 0·5, 1 and 2 units vitamin D respectively. The results were estimated in the usual way. The values obtained for the control animals are those given in Table I and summarised in the curves *A* and *B* in Figs. 1 and 2. Litter 688 was the only litter which might be included in the "moderate" group. The animal in this litter which received no vitamin D gave a value of three degrees of healing, a figure which is on the border line of our arbitrary division between "moderate" and "slight." For this investigation, therefore, the litters were divided into two groups only—those showing "slight" and those showing "severe" rickets. Litter 688 was included in the first group. The results are summarised in Table VI.

Table VI.

	Degree of healing shown by rats which received							
Litters	Diet I (Ca : P = 4 : 1)	Diet II (Ca : P = 4 : 2)	Diet III (Ca : P = 4 : 3)	Diet IV (Ca : P = 4 : 4)	Diet I + 0·25 unit vitamin D daily	Diet I 0·5 unit + vitamin D daily	Diet I + 1·0 unit vitamin D daily	Diet I + 2·0 units vitamin D daily
	"Slight" rickets group.							
563	4·0	6·0	6·0	6·0	6·0	5·75	6·0	6·0
573	4·5	5·0	6·0	6·0	6·0	6·0	6·0	6·0
653	4·0	4·0	6·0	6·0	3·0	3·5	4·75	6·0
688	3·0	6·0	6·0	6·0	5·5	5·5	5·0	6·0
Average value	3·9	5·25	6·0	6·0	5·1	5·2	5·4	6·0
	"Severe" rickets group.							
583	0	5·0	*5·5	4·75	3·25	4·0	6·0	6·0
589	0	4·0	4·5	4·75	3·0	5·0	4·0	4·75
620	0·5	4·25	6·0	5·75	4·5	5·0	5·0	6·0
622	0	6·0	6·0	6·0	4·5	4·25	5·5	6·0
644	0	4·75	6·0	6·0	5·5	4·0	4·25	6·0
700	0	5·0	6·0	6·0	2·0	4·0	5·0	5·0
Average value	0·08	4·8	5·7	5·5	3·8	4·4	5·0	5·6

* Average of values from two rats.

It will be seen that in the "slight" rickets group, diets III and IV both produced complete healing of rickets. In other members of the same litters a daily dose of 2 units of vitamin D given in addition to diet I also produced complete healing, whereas lower doses of the vitamin produced only partial healing. Decreasing the ratio of calcium to phosphorus from 4 : 1 to 4 : 3 is, therefore, equivalent to giving a daily dose of 2 units vitamin D. The "severe" rickets group shows a similar result. Diet III gave an average value of 5·7 degrees of healing and 2 units vitamin D gave an average value of 5·6 degrees of healing. These figures are too high to be found on the sensitive part of the curve for rats showing severe rickets, but they confirm the result given above that diet III is equivalent in effect to a daily dose of 2 units vitamin D. In the "severe" rickets group, diet IV, in which the ratio of calcium to phosphorus is approximately 1, gave a value for the average degree of healing slightly less than that given by diet III. This result may be due to the variation in animals or it may indicate that a calcium to phosphorus ratio of 4 : 3 is the optimum for curing severe rickets. Any further increase in the proportion of phosphorus would then hinder the cure and tend to produce the condition known to be brought about by diets in which the ratio P/Ca is high.

Further comparisons were made by referring the values for the degree of healing produced by diet II to curves *A* and *B*, which were drawn from the animals which had received graded doses of vitamin D in these particular litters. The corresponding abscissae gave the equivalent dose of vitamin. Thus in the "slight" rickets group, diet II produced 5·25 degrees of healing. Reference to curve *A*, Fig. 1, shows that 5·25 degrees of healing would be given by a daily dose of 0·40 unit vitamin D. Diet II is, therefore, equivalent in antirachitic effect to a daily dose of 0·40 unit vitamin D. In the "severe" rickets group, diet II produced 4·8 degrees of healing. Reference to curve *B*, Fig. 2, shows that this value would be given by a daily dose of 0·70 unit vitamin D. This result is higher than that obtained from the "slight" rickets group. In order to determine whether the difference was due entirely to experimental error, results from 5 more litters were examined. The members of these litters were grouped so that at least 1 rat in every litter received one of the following diets—diet I, diet II, diet III, diet IV, diet I + 0·5 unit vitamin D daily, diet I + 1·0 unit vitamin D daily. The rats receiving vitamin D in these litters were not originally included in curves *A* and *B* since some of the doses of vitamin D were not represented in every litter. The effect of the diets was determined by a direct application of Dyer's method for estimating the potency of any unknown substance. The results were referred to the general curves in Fig. 3. Full details of the degree of healing obtained with the diets and with daily doses of 0·5 unit and 1·0 unit vitamin D are given in Table VII. The average values for diets III and IV were too high for the calculation of their relative potencies by means of the curves. The values for diet II were, however, referred to curves I and III (Fig. 3) and the corresponding abscissae were compared with those obtained from 0·5 unit and 1·0 unit vitamin D. The results

Table VII.

Litter	Diet I (Ca : P =4 : 1)	Diet II (Ca : P =4 : 2)	Diet III (Ca : P =4 : 3)	Diet IV (Ca : P =4 : 4)	Diet I + 0·5 unit vitamin D daily	Diet I + 1 unit vitamin D daily
	Degree of healing shown by rats which received					
	"Slight" rickets group.					
461	*4·25	*5·6	*6·0	*5·0	5·75	6·0
463	3·75	3·25	4·0	6·0	*4·6	*4·6
465	4·0	5·25	5·0	3·0	*2·0	*4·6
486	3·0	4·0	6·0	5·0	2·0	5·0
563	4·0	6·0	6·0	6·0	5·75	6·0
573	4·5	5·0	6·0	6·0	6·0	6·0
653	4·0	4·0	6·0	6·0	3·5	4·75
688	3·0	6·0	6·0	6·0	5·5	5·0
Average value	3·8	4·9	5·6	5·4	4·4	5·2
	"Severe" rickets group.					
483	*0·5	4·9	*5·5	*5·9	5·25	6·0
583	0	5·0	*5·5	4·75	4·0	6·0
589	0	4·0	4·5	4·75	5·0	4·0
620	0·5	4·25	6·0	5·75	5·0	5·0
622	0	6·0	6·0	6·0	4·25	5·5
644	0	4·75	6·0	6·0	4·0	4·25
700	0	5·0	6·0	6·0	4·0	5·0
Average value	0·1	4·8	5·6	5·6	4·5	5·1

* Average of values from two rats.

Table VIII.

Dose with which diet II was compared	Ratio of degrees of healing on Diet II to dose of vitamin D	Ratio of corresponding abscissae obtained from curve	Daily dose of vitamin D equivalent to diet II
	"Slight" rickets group.		
0·5 unit vitamin D	$\frac{4·89}{4·39}$	$\frac{0·28}{0·09}=3·1$	1·5 units
1·0 unit vitamin D	$\frac{4·89}{5·24}$	$\frac{0·28}{0·54}=0·52$	0·52 unit
	"Severe" rickets group.		
0·5 unit vitamin D	$\frac{4·84}{4·50}$	$\frac{1·2}{0·82}=1·5$	0·75 unit
1·0 unit vitamin D	$\frac{4·84}{5·11}$	$\frac{1·2}{1·63}=0·74$	0·74 unit

are shown in Table VIII. In the "severe" rickets group, diet II was found to be equivalent to a daily dose of 0·75 or 0·74 unit vitamin D. These results agree closely with each other and confirm the value 0·70 unit found by direct reference to curve *B* as shown above.

In the "slight" rickets group, diet II was found to be equivalent to 1·5 units vitamin D when compared with one dose of standard and 0·5 unit when compared with the other dose of standard, whereas direct reference to curve *A* gave

the value 0·4 unit. These differences are very much greater than those obtained in the "severe" rickets group and appear to confirm the suggestion of Bourdillon *et al.* [1931] that the variation in response is greater for rats which develop slight rickets than for rats which develop more severe rickets. No reliable information could be gained from such widely different results and we have assumed that the most probable value for the daily dose of vitamin D equivalent to diet II is 0·7 unit, which is the value given by the "severe" rickets group.

This result is of importance in the testing of the vitamin D potency of a substance containing a high proportion of phosphorus. Suppose the unknown substance were incorporated in the diet in sufficient quantity to change the ratio of calcium to phosphorus from 4 : 1 to 2 : 1, then healing equal to that produced by 0·7 unit vitamin D would be due to the change in the ratio of calcium to phosphorus and only additional healing could be attributed to the vitamin D in the unknown substance. In the absence of any figures relating smaller alterations in the ratio Ca/P to equivalent doses of vitamin D, it may be assumed that the relationship between healing and Ca/P ratio is given by a curve somewhat similar to that relating healing and dose of vitamin D. It would then appear desirable that, if the ratio Ca/P is changed from 4 : 1 even to 4 : 1·25, the diet of the control animals should be adjusted so that the ratio is the same as in the diet containing the test substance.

Summary.

Two experiments are described in which graded doses of vitamin D were given to young rats in two series of 10 and of 6 litters respectively. The degree of healing shown by each rat was assessed according to Dyer's scale and curves were drawn relating the average degree of healing to the dose of vitamin D. The curves were similar in shape to Dyer's curves and gave approximately the same results for the potency of unknown substances. The results from these experiments were combined with those of Dyer and divided into three groups as follows:

(*a*) litters which developed "slight" rickets;
(*b*) litters which developed "moderate" rickets;
(*c*) litters which developed "severe" rickets.

A curve relating degree of healing to dose of vitamin D was drawn for each group.

The curves were used to estimate the vitamin D potency of a sample of fish meal and the results were found to agree with those obtained in a prophylactic test based on the percentage of ash in the dry fat-free bones. It was shown that the curative test is more generally useful than the prophylactic test since differences in degree of healing could be evaluated by reference to the curves.

The effect of changing the ratio of calcium to phosphorus in Steenbock's rachitogenic diet was determined by the curative test, using the curves to evaluate differences in the average degree of healing produced by different diets. It was found that changing the ratio from 4 : 1 to 2 : 1 produced an anti-rachitic effect equal to that given by a daily dose of 0·7 unit vitamin D.

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XXV. BACTERIAL METABOLISM.

I. LACTIC ACID PRODUCTION BY HAEMOLYTIC STREPTOCOCCI.

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Detailed study of the metabolism of bacteria reveals the diversity of the chemical activities of different species, and Kluyver [1931] has emphasised the need of the development of "comparative biochemistry" to correlate the chemistry of the different micro-organisms. Since Harden's work at the beginning of the century [1901] a considerable volume of data concerning bacterial metabolism has accumulated. Much of the work is reviewed by Stephenson [1930], and a further contribution has been made recently by Birkinshaw, Charles and Clutterbuck [1931]. Nevertheless relatively little information is available regarding the chemical activities of a number of the organisms commonly found in disease.

In this communication are reported some observations made on the production of lactic acid from glucose in cultures of haemolytic streptococci. These organisms have been selected for study, since not only are they associated with many pathological conditions, but they also display biological characteristics of considerable practical and theoretical interest, and are capable of existing in the form of variants of differing chemical structure, serological behaviour, virulence, *etc.* [Todd, 1928, 1930; Todd and Lancefield, 1928]. The metabolism of haemolytic streptococci has been little studied.

Analytical methods.

Preliminary experiments indicated that determinations of glucose and lactic acid supplied all the data required at this stage of investigation.

Since the media to be employed contained small quantities of peptone and other nitrogenous substances, different methods of removing them were investigated. The superiority for this purpose of phosphotungstic acid was established by comparison with sodium tungstate and sulphuric acid, zinc sulphate and sodium hydroxide, and lead acetate and sodium phosphate. The superiority of phosphotungstic acid was due to the immediate, rapid filtrability, and the precipitation of considerable amounts of nitrogenous substances.

Glucose was determined in the phosphotungstic acid filtrate, by the Hagedorn-Jensen [1923] method, as modified first by Hanes [1929] and subse-

quently by Hulme and Narain [1931]. Lactic acid was determined in the filtrate after removing carbohydrates, by shaking with copper sulphate and lime [Van Slyke, 1917]. The method for lactic acid determination was that of Friedemann, Cotonio and Shaffer [1927] based on that of von Fürth and Charnass [1910]. The acetaldehyde formed by oxidation of the lactic acid was removed in a current of air passing through an efficient reflux water-cooled double-surface condenser, and was absorbed in 1 % sodium bisulphite in a 7 × 1 boiling-tube. This results in a 97 % absorption of acetaldehyde [Clausen, 1922].

The *N*/75 sodium thiosulphate used in the glucose determination was standardised by means of a standard potassium iodate solution, and the same sodium thiosulphate solution was used to standardise the *N*/100 iodine used in the lactic acid determination. In this way a cross check on the methods used for the determination of glucose and its metabolic product was obtained. It was satisfactory to note that the "blanks" obtained using the peptone, *etc.*, employed were small [*cf.* Schmidt, 1931], although this was not of great importance, since the results are obtained by difference of the analytical figures of the cultures before and after growth of the organisms. Analyses of the cultures were conducted as follows.

20 cc. of culture filtrate were pipetted into a 100 cc. measuring flask and 1 cc. of 20 % phosphotungstic acid (containing 5 % sulphuric acid) was added. In highly buffered media from 1 to 4 cc. of *N* H_2SO_4 was added also. The volume was made up to 100 cc. and the precipitate was immediately filtered off on a folded paper giving a perfectly clear filtrate (filtrate α). Portions of this, preferably containing 1 to 3 mg. of glucose, were taken for glucose determination. 20 cc. of filtrate α were transferred to a 100 cc. graduated flask, 2 cc. each of 10 % copper sulphate solution and 5 % lime suspension were added, and the volume was made up to 100 cc. After shaking at intervals for at least 30 minutes the suspension was filtered off, yielding the clear filtrate β. Portions of filtrate β were taken for lactic acid determination, using preferably a sample containing 4 to 5 mg. of lactic acid, corresponding to some 10 cc. of *N*/100 iodine solution.

The efficacy of the analytical procedure was examined by means of the following experiments. Duplicate samples of a pure glucose solution each containing 2·00 mg. of glucose were taken and found equivalent to 4·90 and 4·92 cc. respectively of 0·01316 *N* sodium thiosulphate. This gives a mean value of 2·02 mg. of glucose, an error of 1 %. Duplicate determinations were always found to be in good agreement. Glucose was added to the medium used in the metabolism experiments reported later and the glucose content of the phosphotungstic acid filtrate was determined. The medium itself had a glucose equivalent of 0·43 mg. per 1 cc., and the amount of glucose added was 10·25 mg. per 1 cc. The amount of glucose present was determined as 10·95 mg. per 1 cc. (calculated value 10·68 mg.), a recovery of 102·5 %. Glucose added to peptone water was recovered quantitatively in the phosphotungstic acid filtrate; the phosphotungstic acid filtrate of the peptone itself was equivalent to 0·005 g.

per 100 cc. of 0·2 % peptone solution. Addition of 2·5 mg. of lactic acid to 2·0 mg. of glucose had no effect on the glucose equivalent.

Lactic acid determinations by the method described were also found to proceed satisfactorily. Duplicate determinations on 1 cc. samples of a 0·85 % solution of zinc lactate in dilute sulphuric acid gave values of 6·0 and 6·2 mg. respectively of lactic acid (calculated value 6·3 mg.) thus giving a recovery of 97 %. In further experiments, owing to the insolubility of the zinc salt, a sodium lactate solution of determined lactic acid content was used. 87·8 mg. of lactic acid were added to 20 cc. of the culture medium described later which itself had a lactic acid equivalent of 10 mg. The phosphotungstic acid filtrate was prepared and this was shaken with copper sulphate and lime as described above. The result of the analysis indicated a lactic acid content of 100·7 mg. (calculated value 97·8 mg.), a recovery of 103 %. The same percentage recovery of lactic acid was obtained from peptone water precipitated with phosphotungstic acid. Neither precipitation of nitrogen compounds with phosphotungstic acid nor removal of carbohydrates by treatment with copper sulphate and lime resulted in any detectable loss of lactic acid. A zero "blank" value was obtained from a solution containing 50 mg. of glucose which had been shaken with copper sulphate and lime, filtered and subjected to lactic acid determination.

It is evident from the figures quoted that the analytical procedure is accurate and efficient as well as being comparatively rapid and simple.

Culture media.

Haemolytic streptococci require a rather carefully constituted medium for good growth, but it was found that excellent growth was obtained in a medium consisting of broth diluted ten times in a buffered salt solution containing glucose. The broth added to the salt mixture was a meat infusion containing 2 % of proteose peptone, and had not been autoclaved but was sterilised by filtration [Hewitt, 1930] in order to avoid the deleterious effects of over-heating [Hartley and Hartley, 1922; Whitehead, 1926]. The salt solutions were made with A.R. chemicals and contained ammonium sulphate as a source of nitrogen. In the first case, phosphate was used as a buffer, but subsequently bicarbonate was employed, as its buffering power is superior. The method of preparation of the phosphate medium was different from that of the bicarbonate media.

Phosphate medium (M_2).

$(NH_4)_2SO_4$ 5 g.	KCl 1 g.
$Na_2HPO_4.12H_2O$ 8 g.	$MgSO_4.7H_2O$ 0·01 g.

Tap water 900 cc.

The p_H was 7·8. The solution was autoclaved, and 100 cc. of broth and 50 cc. of steamed 10 % glucose solution were added. The p_H was then 7·2. The medium was pipetted into sterile plugged 7 × 1½ in. tubes in 90 cc. lots, and

incubated to ensure that sterility had been maintained. The medium contained 0·575 % of glucose (Hagedorn-Jensen).

Bicarbonate media (M_3, M_4, M_5).

	M_3	M_4 and M_5
Glucose	6 g.	10 g.
$(NH_4)_2SO_4$	5	5
$NaHCO_3$	4	10
KCl	1	1
$MgSO_4, 7H_2O$	0·01	0·01
0·02 % phenol red	3 cc.	3 cc.
Tap water	900	900

Carbon dioxide was passed into the solutions until a p_H of about 6·8 was reached. Then 100 cc. of broth were added and the medium was filtered immediately through a Pasteur-Chamberland F candle. The medium was pipetted into sterile plugged 7 × 1½ in. tubes and a cap of cellophane was secured over each plug with an india-rubber band in order to minimise diffusion of carbon dioxide. After incubation to ensure sterility and at the time of inoculation with the organisms investigated the cellophane caps were discarded; the p_H was then about 7·6. The glucose contents (Hagedorn-Jensen) of the bicarbonate media were: M_3 0·673 %, M_4 1·125 % and M_5 1·080 %.

Experimental procedure.

The tubes containing 90 cc. of medium were inoculated with 0·5 cc. of 24-hour broth cultures, and after from 18 to 60 hours' incubation at 37° they were removed from the incubator. The cultures were filtered through Seitz filters before being analysed. Experiments were conducted under aerobic conditions, but in some cases extra aeration was effected by continuous gentle agitation of the culture, and in three experiments the cultures were incubated anaerobically in a McIntosh and Fildes jar, from which the air had been displaced by hydrogen in the usual way. The haemolytic streptococci used were a laboratory stock Dochez-B 7 strain and Aronson-Schnitzer and Dochez-E 14 strains provided by Dr E. W. Todd. In the case of the last two strains the variants, matt virulent (M.V.), matt attenuated (M.A.) and glossy (G.) were also kindly provided by Dr Todd.

In every case the cultures grew well, especially in the bicarbonate media. The superiority of the bicarbonate medium over the phosphate medium would be expected from the more efficient buffering qualities of the former under the conditions ruling in the cultures. In the phosphate medium there was not more than 0·2 % of glucose breakdown, and the reaction reached p_H 5·5, whilst in the bicarbonate medium as much as 1 % of glucose was broken down, but the p_H did not fall below 6·3. In bicarbonate media an exceptionally heavy growth of organisms was observed despite the low concentration of complex nitrogenous nutrient material in the medium. As observed by Foster [1921]

the limiting factor in glucose breakdown by haemolytic streptococci is frequently the acidity developed in the culture. Since as much as 0·8 % of lactic acid was formed in some cultures it would appear that the lactate ion has but little antiseptic action.

Lactic acid production under different conditions.

The results of the experiments on lactic acid production are summarised in Table I and it will be observed that under the wide variety of conditions employed the yields of lactic acid from the glucose decomposed lay between

Table I. *Lactic acid production from glucose by haemolytic streptococci.* (Results are expressed in g. per 100 cc. of culture.)

Exp. no.	Medium	Strain	Conditions	Incubation time hrs.	Glucose disappeared g.	Lactic acid formed g.	Lactic acid formed / Glucose disappeared %
S 2	Phosphate M_2	Dochez-B 7	Aerobic	18	0·179	0·136	76
S 5	,,	,,	,,	42	0·177	0·136	77
S 8	,,	,,	,,	60	0·190	0·133	70
S 3	,,	,,	Aerated	18	0·174	0·133	76
S 6	,,	,,	,,	42	0·195	0·132	68
S 9	,,	,,	,,	60	0·182	0·127	70
S 4	,,	,,	Anaerobic	18	0·179	0·130	73
S 7	,,	,,	,,	42	0·172	0·133	77
S 10	,,	,,	,,	60	0·175	0·129	74
S 11	Bicarbonate M_3	,,	Aerobic	24	0·500	0·399	80
S 14	,,	,,	,,	48	0·501	0·373	75
S 12	,,	,,	Aerated	24	0·512	0·421	82
S 13	,,	,,	,,	48	0·519	0·399	77
S 18	Bicarbonate M_4	,,	Aerobic	24	1·030	0·820	80
S 20	,,	Aronson; M.A.	,,	24	0·600	0·478	80
S 21	,,	,, M.V.	,,	48	1·080	0·798	74
S 22	,,	,, M.A.	,,	48	0·860	0·681	79
S 23	,,	,, G.	,,	48	0·310	0·252	83
S 24	Bicarbonate M_5	,, M.V.	,,	48	0·990	0·814	82
S 25	,,	,, G.	,,	48	0·505	0·421	83
S 26	,,	Dochez-E. 14; M.V.	,,	48	1·007	0·848	84
S 27	,,	,, G.	,,	48	0·827	0·706	85
S 28	,,	,, M.V.	,,	24	0·800	0·641	80
S 29	,,	,, M.A.	,,	24	0·215	0·176	82

the limits 68 % and 85 %, with a mean of 78 %. This percentage seems remarkably constant, when consideration is made of the possible sources of variation. In those experiments in which the glucose breakdown was less than 0·2 g. per 100 cc. of culture the experimental errors must be appreciable.

There seems to be little difference between the results obtained from aerobic, aerated and anaerobic cultures, or between incubation for 18 and 60 hours. Different strains of haemolytic streptococci and different variants of the same strain appear to produce practically the same proportion of lactic acid from the glucose decomposed. Although the average proportion of lactic acid produced is a little higher in bicarbonate broth (80 %) than in phosphate broth (73·5 %), the fact that some four times as much glucose is broken down in the

former medium, and the consequently greater experimental accuracy render doubtful the significance of this difference.

It may be taken therefore that under the varying conditions employed in these experiments about three-quarters of the glucose disappearing from the cultures may be recovered in the form of lactic acid.

Other metabolic products.

Careful examination of products of glucose metabolism, other than lactic acid, have not yet been made. In any case the concentration of these cannot be great, the amounts of glucose unaccounted for in the present experiments varying from 0·04 % to 0·28 %.

Harden and Walpole [1906], and Birkinshaw, Charles and Clutterbuck [1931] have shown that considerable amounts of 2 : 3-butyleneglycol are produced by certain bacteria, and the possibility of this occurring in the case of haemolytic streptococci was investigated.

Prof. Raistrick and Dr Birkinshaw kindly provided a sample of butyleneglycol, and it was found that the glycol gave two molecules of acetaldehyde with a 90 % yield in the lactic acid apparatus.

5 cc. of the pooled cultures S8, S9 and S10 containing 0·13 % of lactic acid were brought to p_H 7·4 and extracted with ether in a continuous extraction apparatus for 5 hours. Oxidation in the lactic acid apparatus in the usual manner revealed a concentration of less than 0·002 % of butyleneglycol.

For the detection of volatile acids 75 cc. of the pooled cultures S11 and S12, containing 0·5 % of lactic acid, were acidified with 1 cc. of 85 % phosphoric acid, and 60 cc. were distilled off at atmospheric pressure. The distillate required 1·95 cc. of $N/10$ sodium hydroxide to give a pink colour with phenolphthalein so that the percentage of volatile acids in the culture appeared not to exceed 0·015 % (calculated as acetic acid) and was probably less, since lactic acid is itself somewhat volatile [*cf.* Birkinshaw, Charles and Clutterbuck, 1931].

Glucose breakdown by different variants.

The fact that it is possible, by suitable choice of media and conditions of cultivation, to select haemolytic streptococcus variants of different pathogenicity, *etc.* [Todd, 1930], suggests that the metabolism of such variants may differ. As seen in Table I the proportion of lactic acid formed from the glucose broken down appears not to vary in the case of the different variants, but the rate of glucose breakdown by the different variants varies greatly as shown below.

In each case most glucose disappeared in the matt virulent cultures and least in the glossy cultures, the matt attenuated cultures being intermediate. The different behaviour of the different variants was readily seen on inspection of the 24-hour cultures in bicarbonate broth containing phenol red. The matt

Table II. *Glucose breakdown by variants of haemolytic streptococci.*
(Results are expressed in g. per 100 cc.)

	Aronson-Schnitzer strain		Dochez-E 14 strain	
	Exp. I	Exp. II	24 hours' incubation	48 hours' incubation
Matt virulent	1·080	0·990	0·800	1·007
Matt attenuated	0·860	—	0·215	—
Glossy	0·310	0·505	0·065	0·827

virulent cultures had become yellow and carbon dioxide was being liberated with effervescence, whilst the glossy cultures retained a slight pinkish tint and displayed no effervescence.

The more rapid glucose breakdown is associated with heavier growth, an exceptionally bulky deposit of bacteria being observed in the matt virulent cultures. As shown in Table II the difference between the variants is less after longer incubation, since the glucose breakdown in the matt virulent culture slows down as the culture becomes more acid, and as most of the glucose (93 %) has been decomposed after 48 hours' incubation.

Discussion.

Of the glucose disappearing from haemolytic streptococcus cultures the percentage which may be recovered in the form of lactic acid (about 78 %) remains remarkably constant over a wide range of cultural conditions. The cleavage of glucose by haemolytic streptococci appears to be a much simpler process than in the case of many organisms. In the fermentation of glucose by *B. coli*, for example, lactic, acetic, formic and succinic acids, ethyl alcohol, carbon dioxide and hydrogen are all liberated in appreciable amounts [Harden, 1901], and the fermentation products of micro-organisms may be modified, qualitatively or quantitatively, by a variety of conditions including the addition of phosphate, alteration of the oxygen supply [Grey and Young, 1921; Meyerhof and Finkle, 1925; Birkinshaw, Charles, Hetherington and Raistrick, 1931], modification of strain of organisms [Harden and Penfold, 1912] and length of incubation [Grey, 1928]. In contrast with these results no very marked alteration in the percentage of lactic acid formed from the glucose decomposed by haemolytic streptococci was produced by varying

(1) phosphate concentration by addition of 0·8 % sodium phosphate;
(2) p_H range from 7·4–5·3 to 7·4–6·9;
(3) conditions of oxygen supply from extra aeration to anaerobiosis;
(4) length of incubation from 18 to 60 hours;
(5) amount of glucose fermented from 0·18 % to 1·08 %;
(6) strain and variants of organisms.

These facts lend considerable support to Harden and Penfold's [1912] suggestion that lactic acid formation in bacterial fermentation is a separate process from the production of alcohol, acetic acid, carbon dioxide and

hydrogen. The absence of catalase from haemolytic streptococci has pronounced effects on the oxidation-reduction potentials of their cultures [Hewitt, 1931], and the lack of cytochrome and other oxidising systems and enzymes is reflected in the products of glucose metabolism. The proportion of lactic acid formed is higher than with many organisms, since the amount of lactic acid formed in many cases does not exceed 50 % of the glucose decomposed. Accumulation of lactic acid occurs in the case of cancer cells.

The present results differ widely from those of Foster [1921] who found that of the glucose decomposed by haemolytic streptococci only some 30 to 40 % was accounted for as lactic acid and from 5 to 15 % as volatile acids. Foster used undiluted broth containing added glucose as culture medium and only some 0·15 % of glucose disappeared from the cultures. Thus a very high proportion of the metabolic products must have come, not from the glucose, but from other constituents of the medium. In the experiments now reported the medium consisted of broth diluted in ten volumes of a glucose-inorganic salt solution, and up to 1·08 % of glucose disappeared from the cultures, hence a more accurate picture of glucose metabolism must be obtained from the results. The difference in analytical methods is possibly of greater moment than that of the media. The method used by Foster for determining lactic acid offers many possibilities of error and his duplicate experiments differed considerably.

A further point of interest in the results is the difference in the amounts of glucose fermented by the different variants of the same strain of haemolytic streptococci. The matt variants contain a type-specific precipitating substance and may be virulent or attenuated, whilst the glossy variant contains no type-specific substance and is avirulent [Todd, 1928; Todd and Lancefield, 1928; Lancefield and Todd, 1928]. Since these variants may be selected artificially by cultivation under appropriate conditions [Todd, 1930] it is evident that their behaviour towards the surrounding medium must differ. It was found that glucose cleavage was effected much more rapidly in matt virulent cultures than in glossy cultures, the matt attenuated variant being intermediate in its rate of glucose breakdown. The difference, in the case of the variants of the two strains investigated, was strikingly illustrated in 1 % glucose-bicarbonate medium containing phenol red. After 24 hours' incubation the matt virulent cultures were yellow in colour and gave off gas freely, whilst the glossy cultures retained a pink tint and showed no sign of effervescence. The gas liberated in the matt virulent cultures was carbon dioxide produced from the bicarbonate by the action of the acid fermentation products. These observations provide a striking example of the inter-relation of the chemical activities and biological behaviour of bacteria and suggest the possibility of differentiating variants by inspecting the appearance of the cultures in appropriate glucose media [*cf.* Arkwright, 1930].

Differences in the aerobic glycolysis of the different types of pneumococci have been described by Finkle [1931], but this worker found no consistent difference between the rough and smooth variants. With one type the smooth

variant gave more aerobic glycolysis, with a second type the rough variant gave more aerobic glycolysis and in a third case neither variant showed aerobic glycolysis.

It must be emphasised, however, that this worker studied, not growing cultures, but washed suspensions of bacteria. Apart from the difference between measuring manometrically the acid liberated over a short period of time from a heavy suspension of washed cells, and observing the glucose breakdown in an actively growing culture over a period of 24 or 48 hours, there is the further observation, made by Kendall, Friedemann and Ishikawa [1930], that it is difficult to obtain reproducible observations on the rate of chemical changes produced by washed suspensions of bacteria owing to the variable damage caused to the organisms by the washing and centrifuging processes. Finkle's results are not therefore strictly comparable with those described in this communication.

SUMMARY.

1. Excellent growth of haemolytic streptococci was obtained in a salt solution buffered with bicarbonate and containing glucose and 10 % of meat broth.

2. About three-quarters of the glucose broken down in haemolytic streptococcus cultures yielded lactic acid, and this proportion remained remarkably constant over a range of cultural conditions, thus indicating a relatively simple fermentation process.

3. Glucose breakdown was much more rapid in matt virulent cultures than with the glossy variants in the case of the strains investigated. Metabolic differentiation of bacterial variants is thus indicated.

The author is indebted to Dr R. G. White and Dr E. W. Todd for their help and encouragement.

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XXVI. THE GLYCERIDE FATTY ACIDS OF FORAGE GRASSES.

I. COCKSFOOT AND PERENNIAL RYEGRASS.

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Although grasses constitute the chief food of most herbivorous animals we believe we are correct in stating that no knowledge exists of the amount of true fats and phosphatides which they contain. The agricultural chemist, it is true, generally determines the "ether extract" when analysing samples of grasses, but it has been realised since the pioneer work of Kellner that this value does not by any means represent true fat, and in nutrition experiments it is generally converted into the corresponding "starch equivalent."

During the past two years a large amount of data has been collected in this laboratory concerning the composition of the ether extract of various forage grasses, and in particular the amount of true fats, phosphatides, unsaponifiable material (including sterols), waxes and pigments has been determined. Before presenting the general results of this research, which are of agricultural as well as chemical interest, it is proposed to describe in some detail the more extensive investigations which have been made into the chemical constitution of the above-mentioned groups of substances present in the well-known forage grasses, cocksfoot (*Dactylis glomerata*) and perennial ryegrass (*Lolium perenne*). Details of the wax investigation have already been published [Pollard *et al.* 1931]; the present paper deals with the glyceride fatty acids.

Preparation of the glyceride fatty acids. The ether extract from the fresh grass was prepared by the method of Chibnall and Channon [1927, 1] as recently modified in this laboratory [Pollard *et al.* 1931]. The extract was concentrated until it contained about 25 % of fatty material, when 2 volumes of warm acetone were added. The mixture was allowed to stand in the ice-chest for some hours, and the precipitated waxes and phosphatides were removed by filtration. The acetone-ether filtrate, which contained the glycerides, unsaponifiable material, pigments, *etc.*, was then treated by the method of Chibnall and Channon [1927, 2] to obtain the unsaponifiable material and the glyceride fatty acids.

The glyceride fatty acids of cocksfoot.

Batch P. 33 kg. of fresh grass (4·9 kg. dry wt.) gave 283 g. of ether extract, from which were obtained 107·5 g. of a semi-solid mixture of fatty acids. The yield was therefore 38 % of the ether extract and 2·2 % of the dry weight of the grass. The acids had an iodine value (I.V.) of 170 and a mean molecular weight of 278. Before saponification the glyceride fraction contained only 2·3 % of free fatty acids.

First separation of the saturated and unsaturated acids.

Chibnall and Channon [1927, 2] attempted to prove the presence of oleic acid in the glyceride fatty acids from cabbage leaves by using the method of Levene and Rolf [1922], which depends on the difference in solubility in various solvents of the barium and lead soaps of the saturated acids, oleic acid and the more unsaturated acids. No evidence for the presence of oleic acid was, however, obtained.

In the present case 107·5 g. of fatty acids were converted into the barium soaps and then treated with benzene containing 5 % of 95 % alcohol. The insoluble portion, which should have contained the barium soaps of the saturated acids and of oleic acid, was freed from barium and the acids were converted to the lead soaps. These were then treated with ether, in which solvent the soaps of the saturated acids are fairly insoluble and those of oleic acid are soluble. The soluble fraction was freed from ether by evaporation, the dry soaps were dissolved in hot benzene, and lead was removed by hydrogen sulphide. The recovered acids were then again put through the barium and lead soap treatments successively. The final fraction, which should have contained any oleic acid, weighed only 1·5 g., and had an I.V. of 192. It was clear from this result that the amount of oleic acid in the original mixed fatty acids must have been very small if, indeed, the acid was present at all. The lead soaps insoluble in ether gave 7·2 g. of solid acid of M.P. 49–50° and I.V. 12. Assuming that the I.V. is due to a small proportion of the unsaturated acids mentioned below the corrected amount of saturated fatty acid was 6·7 g., or 6·2 % of the original mixture. The unsaturated acids recovered from the various fractions of lead and barium soaps, together with the so-called oleic acid fraction of 1·5 g. mentioned above, were collected, and gave 82·5 g. of liquid acids of I.V. 185. This corresponds to 76·7 % of the original acid mixture, showing that there had been a loss in the series of operations of approximately 17 %. It will be shown later that this falls almost entirely on the unsaturated acids.

Identification of the unsaturated acids by bromination.

Hexabromo-derivatives. 10·4 g. of the unsaturated fraction mentioned above were brominated in anhydrous ether at 0° in the usual way. 5·89 g. of insoluble bromo-derivatives were formed, which were completely soluble in

boiling benzene, showing the absence of octabromo-derivatives. On cooling the benzene solution 5·1 g. of hexabromostearic acid crystallised out. (Found: M.P. 180–180·5°: C, 28·8; H, 4·1; Br, 63·0 %. $C_{18}H_{30}O_2Br_6$ (M.P. 180–181°) requires C, 28·5; H, 4·0; Br, 63·3 %.) The amount of α-linolenic acid was therefore 21·2 % of the unsaturated fraction or 16·3 % of the original fatty acid mixture.

Tetrabromo-derivatives. The bromination products soluble in ether were freed from excess bromine by treatment with a solution of sodium thiosulphate, the ethereal solution dried with anhydrous sodium sulphate, the ether removed by evaporation and the residue treated with cold light petroleum (B.P. 40–60°). The insoluble portion weighed 8·9 g. It was soluble in hot solvents, but on cooling separated as an oil, and no crystalline tetrabromostearic acid, M.P. 114°, could be obtained. Analysis showed that it was probably a mixture of isomeric tetrabromostearic acids. (Found: C, 34·3; H, 4·9; Br, 52·6. $C_{18}H_{32}O_2Br_4$ requires C, 36·0; H, 5·4; Br, 53·3 %.) That the acid, M.P. 114°, derived from ordinary linoleic acid (9 : 12-octadecadienoic acid) was present, although it could not be obtained crystalline, was shown by conversion of the tetrabromo-acids into the corresponding tetrahydroxy-acids. 1 g. of the tetrabromo-fraction was reduced by Rollet's method [1909] and the resulting unsaturated acids oxidised by the method of Haworth [1929]. 0·2 g. of mixed α- and β-sativic acids was obtained, M.P. 155–163°. (Found: C, 62·1; H, 10·4. $C_{18}H_{36}O_6$ requires C, 62·0; H, 10·4 %.) In Table I this fraction is referred to as α-linoleic acid.

Bromo-derivatives soluble in light petroleum. This fraction should have contained any dibromostearic acid, any saturated acid which still remained in the unsaturated fraction now under discussion, and any tetra- and hexa-bromo-acids derived from corresponding unsaturated acids. Following the nomenclature of Kaufmann and Keller [1931] we shall refer to these unsaturated acids as β-linoleic and β-linolenic acids respectively. Both are probably mixtures of unknown isomerides, which have the property in common of giving —under the usual well-defined conditions—bromo-derivatives which have solubilities different from those of the known α-linoleic and α-linolenic acid respectively.

The bromine content of this light petroleum-soluble fraction was 45·8 %. To remove any residual saturated acid, 8·0 g. of the products were converted to the lead soaps, which were then dissolved in a small volume of warm ether and left overnight in the ice-chest. The insoluble soaps yielded 0·6 g. of saturated acid. This amount represents 5·7 % of the unsaturated fraction. The soluble lead soaps yielded a bromo-product giving the following analysis: C, 38·1; H, 5·7; Br, 51·5 %. It is shown later that Bertram's method of oxidation gave a higher value (12 % as against 5·7 %) for the saturated acids than the lead soap precipitation methods, so that the above fraction probably still contained saturated acid. As tetrabromostearic acid has 53·3 % Br, it follows that this fraction must have contained some hexabromostearic acid and but very

little dibromostearic acid. To investigate the fraction further 1 g. was debrominated by the method of Rollet [1909] and the resulting unsaturated acids oxidised by alkaline permanganate [Haworth, 1929]. A small amount of mixed α- and β-sativic acids was isolated, showing the presence of a tetrabromostearic acid in the bromo-fraction, but no dihydroxystearic acid was obtained, again showing the probable absence of oleic acid from the original mixed fatty acids.

The bromination experiments therefore suggest that the unsaturated fraction of the fatty acids had the composition shown in Table I. It is assumed

Table I. *Composition of the glyceride fatty acids of cocksfoot as suggested by bromination experiments.*

	Unsaturated fraction %	Original mixed fatty acids %
Saturated acids (Bertram)	12	15
Oleic acid	—	—
α-Linoleic acid	38·5	29·5
β-Linoleic acid	21·0	16
α-Linolenic acid	21·0	16
β-Linolenic acid	7·5	6
Total	100·0	82·5*

* There was a loss of 17 % in the initial fractionation into saturated and unsaturated acids.

that oleic acid is absent, and the amounts of β-linoleic acid and of β-linolenic acid have been calculated from the original I.V. (185·3) of the unsaturated fraction.

Identification of the unsaturated acids by oxidation.

1. *Method of Lapworth and Mottram [1925].* This method is known to give 95 % yields of dihydroxystearic acid from oleic acid, and was applied in the present case with the object of demonstrating, if possible, the presence of oleic acid in the unsaturated fraction. 11 g. of this fraction were dissolved in 1 litre of a 1 % solution of sodium hydroxide, which was then poured into 8 litres of ice-cold water. 800 cc. of 1 % solution of potassium permanganate were added quickly with stirring, and after 5 minutes a stream of sulphur dioxide was passed in until the mixture was colourless. Concentrated hydrochloric acid (150 cc.) was then added, and the white flocculent precipitate filtered off, washed, and dried *in vacuo* at 100°. Treatment with light petroleum (B.P. 40–60°) removed 1·54 g. of solid acid, M.P. 35–40°. The residue (M.P. 145°), weighing 0·9 g., was extracted with ether, which removed 0·4 g. of a gum from which no dihydroxystearic acid could be obtained. It is improbable therefore that any oleic acid was present in this unsaturated fraction. The material insoluble in both light petroleum and ether (0·45 g.) melted indefinitely at 163–165° with previous softening at 145–150°. It was a tetrahydroxystearic acid. (Found: C, 61·6; H, 10·4. $C_{18}H_{36}O_6$ requires C, 62·0; H, 10·4 %.) 0·17 g. was heated with ethyl acetate and filtered hot. On cooling α-sativic acid (0·06 g.) crystallised out, M.P. 159–160°. The insoluble residue (0·1 g.) was β-sativic acid, M.P.

171·5–172·5°. According to Meyer and Beer [1912] α- and β-sativic acids fractionated in this way melt at 163° and 173° respectively.

The aqueous filtrate, after removal of the above-mentioned white flocculent precipitate, amounted to approximately 12 litres. 8 litres of this were concentrated *in vacuo* to 1 litre and filtered hot. The residue on the filter-paper was boiled with ethyl alcohol and again filtered hot. On cooling the filtrate deposited 0·25 g. of white material, M.P. 202°. It was recrystallised from water; M.P. 203–4°. (Found: C, 56·9; H, 9·2. $C_{18}H_{36}O_8$ requires C, 56·8; H, 9·5 %.) Hazura records a melting-point of 203–5° and Krzizan of 204–5° for linusic acid [Lewkowitsch, 1921].

2. *Method of Haworth* [*1929*]. This method differs from the former only in certain details of procedure, and was applied to confirm the absence of oleic acid. 9·6 g. of the unsaturated fraction gave insoluble oxidation products which, after extraction with light petroleum as before, yielded on extraction with ether 0·35 g. of a dark gum. No crystalline dihydroxystearic acid could be obtained from this material. (Found: C, 66·6; H, 9·3. $C_{18}H_{36}O_4$ requires C, 68·35; H, 11·5 %.)

These two oxidation methods therefore confirm the presence of linoleic and linolenic acid in the unsaturated fraction, and the probable absence of oleic acid.

Determination of the total saturated fatty acids.

Method of Twitchell [*1921*]. The procedure followed that recommended by Hilditch and Priestman [1931]. Two experiments were carried out, one in which the amount of lead acetate added was only slightly in excess of that required to convert the saturated acids into their lead soaps, and the other in which it was sufficient to convert all the acids present into their lead soaps.

In the first experiment 9·84 g. of the unsaturated fraction were dissolved in 30 cc. of 95 % (by weight) alcohol and 1·2 g. of lead acetate in 70 cc. of boiling 95 % alcohol added. The hot mixture was allowed to cool very slowly to 15–20° and then left overnight in an oven so that the temperature was strictly maintained within these limits. The crystalline lead soaps were then filtered off, washed with 95 % alcohol till the washings no longer became turbid on diluting with a drop of water, and then recrystallised as before from 100 cc. of hot 95 % alcohol containing 0·5 g. acetic acid. 0·63 g. of dark brown solid acid was recovered from the lead soaps; M.P. 48°, I.V. 13. Correcting for the I.V. this amount of acid corresponds to 6·0 % of the unsaturated fraction. In the second experiment the value was slightly higher, 6·8 %. Both of these results are of the same order as that given by the lead soaps in the bromination experiments previously described (p. 220) which was 5·7 %, but are definitely lower than that given by the Bertram oxidation described below.

Method of Bertram [*1927*]. The procedure followed that recommended by Hilditch and Priestman [1931]. 9·63 g. of the unsaturated fraction were dissolved in 200 cc. of a 5 % solution of potassium hydroxide. Water (1300 cc.)

containing 60 g. of potassium permanganate was then added, the temperature being maintained between 35° and 50°. After standing overnight the solution was decolorised with sulphur dioxide and exhaustively extracted with light petroleum (B.P. 60–80°). There was obtained 1·89 g. of a soft brown solid from which 0·34 g. of non-acidic material was removed in the usual way. The acidic products were precipitated twice as the magnesium soaps and on decomposition yielded 1·14 g. of recrystallised acid.

This yield is equivalent to 11·8 % of the unsaturated fraction, which is about 5·6 % higher than the corresponding value given by Twitchell's method. This large difference might be due to (1) the solubility of lead palmitate and higher homologues in alcohol being increased by the presence of relatively larger amounts of lead linoleate and linolenate; (2) the presence of saturated acids lower in the series than palmitic, which are known to be only partially precipitated, if at all, in the Twitchell process; (3) the fact that Bertram's method has given saturated acids lower than palmitic acid by oxidation of unsaturated acids with the double bonds in positions near the end of the carbon chain. It is shown in a later experiment (p. 233) that the amount of shorter acids pre-existing in the original mixed fatty acids was exceedingly small, and analysis does not suggest the presence of shorter acids in the Bertram oxidation products. (Found: C, 75·3; H, 12·6: mol. wt. by titration, 266. $C_{16}H_{32}O_2$ requires C, 74·9; H, 12·6; mol. wt. 256. $C_{18}H_{36}O_2$ requires C, 76·0; H, 12·8 % and mol. wt. 284.) Furthermore the shorter fatty acids are not readily precipitated as the magnesium soaps. We attribute the difference between the Twitchell and Bertram methods therefore to the residual solubility of lead palmitate, and accept the higher value given by the latter method as correct. It is interesting to note that Kaufmann finds that Bertram's method frequently gives results which are 1 to 2 units % higher than those given by the lead soap methods.

Identification of the saturated acids.

8·0 g. of recrystallised acids, M.P. 50°, were esterified with ethyl alcoholic hydrogen chloride in the usual way. The 8·4 g. of crude esters thus obtained were distilled at 0·3 mm. giving four fractions and a residue. Each of these was then saponified, and the recovered acids were crystallised repeatedly from acetone or pyridine. The collected analytical data are given in Table II, and the following conclusions can be drawn.

(*a*) Fractions 1 and 2 consist of nearly pure palmitic acid. There is no evidence of a lower acid (ethyl myristate melts at 10·5–11·5°, and myristic acid has mol. wt. 228).

(*b*) Fraction 5 is a mixture of longer fatty acids of mean molecular weight corresponding to $C_{26}H_{52}O_2$. It is clearly the usual "cerotic acid" which occurs extensively in plant fats and waxes, and which was shown by Francis, Piper and Malkin [1930] to be a mixture. A similar product, of slightly higher mean molecular weight, was found in the wax fraction of the cocksfoot ether extract

Table II. *Fractionation of the saturated acids from cocksfoot.*

Esters				Acids					
Fraction	B.P. at 0·3 mm.	Weight (g.)	M.P. (approx.)	Weight (g.)	Purification by recrystallisation from acetone (A) or pyridine (P)	M.P.	C (%)	H (%)	mol. wt. (titration)
1	124°	1·32	22–22°	1·1	Once from A	60·5–61·5°	74·9	12·7	258
					Five times from A	62·0–62·7°	75·0	12·6	—
2	124°	2·35	21–22°	2·1	Once from A	60–61°	75·15	12·65	258
3	130°	1·31	23–32°	1·2	Once from A	55·5–57·5°	75·1	12·5	263
					Five times from A	54–55°	—	—	—
4	135–145°	1·15	29–32°	1·05	Once from A	52·5–53·5°	75·7	12·6	293
					Three times from A	62·0–63·5°	77·5	13·0	—
5	Not distilled	1·96	38–42°	1·8	Once from A	67–68°	77·4	13·0	—
					Four times from A	72·5–73·5°	78·3	13·3	384
					(followed by)				
					Once from P and twice from A	79·5–80·5°	78·4	13·2	—
Ethyl stearate			34–35°	—	Palmitic acid requires	63°	74·9	12·6	256
Ethyl palmitate			25°	—	Stearic acid „	70°	76·0	12·8	284
Ethyl myristate			10·5–11·5°	—	"Cerotic acid" „	80–82°	78·7	13·2	396

[Pollard *et al.* 1931], and the small amount encountered in the present case may well be due to the residual solubility of the waxes in the ether-acetone used to precipitate the latter in the preparation of the glyceride fraction.

(*c*) Fractions 3 and 4 call for more extended discussion. On repeated recrystallisation the M.P. of the former acid was depressed slightly to 54–55°. It was clearly a mixture and the M.P. suggested 70 % palmitic acid with 30 % stearic acid. Such a mixture however was shown to yield without difficulty a higher-melting fraction when recrystallised three times from acetone. To obtain further evidence six mixtures, all of which had a mean molecular weight and carbon and hydrogen content similar to that of Fraction 3, were prepared, and their melting-points determined. Each was then recrystallised three times from acetone or acetone-pyridine, and the M.P. of the least soluble fraction determined. The data obtained are given in Table III. It will be seen that all the

Table III. *Melting-points of certain mixtures of fatty acids prepared from purified acids.*

No.	% of palmitic acid M.P. 61–61·7°	% of stearic acid M.P. 68–68·5°	% of eicosanic acid M.P. 75–75·2°	% of cerotic acid M.P. 77–78°	M.P. of mixture	M.P. of least soluble fraction after 3 recrystallisations from acetone
1	91	—	—	9	59·5–60·5°	84°
2	67	27	—	6	52·5–54·0°	66·5–68°
3	69	29	—	2	54–55°	60–61°
4	70	30	—	—	55–55·5°	61·5–63·5°
5	78	—	22	—	54·5–55·5°	74·5–75·0°
6	67	27	6	—	53·5–54°	54–54·5°

binary mixtures were readily separated into higher-melting fractions, especially those of palmitic acid with an acid higher in the series than stearic acid. The mixture composed of 67 % palmitic, 27 % stearic and 6 % eicosanic acids was the only one which was in any way analogous to Fraction 3, and there would seem to be no doubt that this represents fairly closely the composition of the latter. In the same way Fraction 4 is probably a similar mixture in which a small amount of "cerotic acid" replaces the eicosanic acid. The evidence from Tables II and III therefore shows that the saturated fraction consists of about 60 % palmitic acid, 20 % stearic acid and 20 % of a mixture of acids containing 20, 22, 24, *etc.*, carbon atoms, of which the higher ones constitute the so-called "cerotic acid."

Thiocyanometric analysis of the fatty acids.

Description of the method. The fact that oleic acid is always assumed to be present in glyceride fatty acid mixtures of the type under discussion made it imperative that more definite evidence for its presence or absence should be obtained. It was therefore decided to apply Kaufmann's thiocyanometric method of analysis, which is being increasingly used in researches into the composition of fatty acid mixtures. A list of references to Kaufmann's work [1925–31] on thiocyanometric analysis is given at the end of this paper, but

the one of chief interest in connection with mixtures containing linolenic acid is that of Kaufmann and Keller [1929]. The method of analysis is based on the observation that, whereas oleic, linoleic and linolenic acids absorb 2, 4 and 6 atoms of halogen, they absorb only 2, 2 and 4 CNS radicals respectively. The thiocyanogen number (CNS v.) is calculated for purposes of comparison as an iodine number (I.V.), and if S, O, L and Ln represent the percentages of saturated, oleic, linoleic and linolenic acids respectively, it can be readily shown that the composition of a mixture of fatty acids can be calculated from the following three equations.

$$S + O + L + Ln = 100,$$

$$O + 2L + 3Ln = \frac{100}{90{\cdot}6} \text{ I.V.},$$

$$O + L + 2Ln = \frac{100}{90{\cdot}6} \text{ CNS v.}$$

The validity of the method depends on Kaufmann's assumption that the CNS v. of linolenic acid is 183. The evidence for this is indirect, as the pure acid has not yet been prepared. This point will be discussed in greater detail after the results of our own analysis have been given.

Application of the method to the unsaturated fraction. The thiocyanogen solution was prepared by Kaufmann's method [1928] and a reaction period of 24 hours was used. The CNS v. was found to be 115·3. It has already been shown that the I.V. of this fraction was 185·3, that the amount of α-linolenic acid was 21·2 % (bromination experiments) and that the amount of saturated acid was 11·8 % (Bertram). Using these values in conjunction with the equations given above, the analysis given in Table IV was obtained. It will be

Table IV. *Thiocyanometric analysis of the glyceride fatty acids of cocksfoot and ryegrass.*

	Batch P. Cocksfoot		Batch N. Cocksfoot	Batch C. Ryegrass	
	Saturated acids by Twitchell's method %	Saturated acids by Bertram's method %	Saturated acids by Twitchell's method %	Saturated acids by Twitchell's method %	Saturated acids by Bertram's method %
Saturated acids	11·1	15·2	10·6	11·9	16·8
Oleic acid	12·5	8·9	16·5	22·5	17·6
Linoleic acid	33·4	29·8	30·9	26·1	21·2
Linolenic acid	25·8	29·5	42·0	39·5	44·4
Total	82·8*	83·4*	100·0	100·0	100·0
α-Linolenic acid by bromination	16·3	16·3	19·5	16·6	16·6

* There was a loss of 17 % in the initial fractionation into saturated and unsaturated acids.

seen that the method postulates the presence of 8·9 % of oleic acid in the original mixed fatty acids. After the evidence obtained from the bromination and oxidation experiments this result was unexpected, and we decided to repeat the analysis on a second sample of cocksfoot glyceride fatty acids.

Analysis of another sample of cocksfoot glyceride fatty acids.

Batch N (fresh weight, 1·84 kg.; dry weight, 276 g.) gave 18·0 g. of ether extract and 6·4 g. of glyceride fatty acids. The I.V. was 184·8 and the CNS v. was 119. 6·0 g. of the acids were separated into saturated and unsaturated acids by Twitchell's method. The weight of solid acids was 0·68 g.; M.P. 52–53°; I.V. 13. Correcting for the small amount of unsaturated acid present the yield of saturated acids was 10·6 %. Bromination of the unsaturated acids in anhydrous ether gave 3·1 g. of hexabromostearic acid, showing that the original mixed fatty acids contained 19·5 % of α-linolenic acid. Unfortunately sufficient original fatty acid was not available for a determination of the saturated acids by Bertram's method. The result of the thiocyanometric analysis is given in Table IV. The presence of a large amount of oleic or some other unsaturated acid containing one double bond is again suggested.

The low I.V. (13) of the saturated fraction obtained by Twitchell's method shows that this cannot have been due to petroselinic (6-octadecenoic) acid, which gives an alcohol-insoluble lead soap.

Discussion.

It will be convenient first of all to summarise in some detail the results of the oxidation and bromination experiments with Batch P.

(1) Bromination showed that 21 % of the unsaturated fraction was α-linolenic acid. From the amount of light petroleum-soluble hexabromo-derivatives it was concluded that the unsaturated fraction also contained about 7·5 % of the so-called β-linolenic acid. This may be a mixture of stereo-isomerides of the α-acid, or perhaps of acids with double bonds in different positions. On oxidation only a small amount of linusic acid was obtained, suggesting that the β-acid had been broken into quite short-chain water-soluble products.

(2) Oxidation gave small amounts of α- and β-sativic acids, showing the presence of α-linoleic acid in the unsaturated fraction. Bromination experiments suggested that β-linoleic acid was present, but on oxidation only short-chain water-soluble products were formed from it.

(3) Oxidation did not give any dihydroxystearic acid melting at either 95° or 132°. If oleic or eläidic acid were present in the unsaturated fraction the amount must have been well under 5 %. No evidence of the production of lauric acid was obtained, so that petroselinic (6-octadecenoic) acid was absent or present only in small amounts. The bromo-derivatives soluble in light petroleum had a bromine content of 51·5 %. If allowance be made for the residual amount of saturated acid known to be present, very little, if any, dibromostearic acid can have been present.

Reverting to the thiocyanometric method of analysis, Kaufmann has adopted two methods to show that linolenic acid takes up thiocyanogen at two

of its three double bonds. (1) He considers what would result if this acid acted towards CNS at one, two, or all three of its double bonds. If it acted at only one, then the CNS v. of a mixture containing oleic, linoleic and linolenic acids could never exceed 91. This was contrary to his experience with many unsaturated oils, hence the linolenic acid must act at more than one double bond. If the linolenic acid were assumed to act at all three double bonds, in the case of many linseed oils a large negative value for oleic acid was obtained. Hence Kaufmann concluded that it must act at two of its double bonds. (2) A sample of linseed oil was taken for analysis. The saturated acids were removed by dissolving the oil in pentane and leaving the solution for some time at $-18°$, when the solid acids crystallised out. The oleic acid was then removed by the lithium soap method of Moore [1919], which is described later (p. 230). The remaining mixture consisted of linoleic and linolenic acids. Measurement of the I.V. enabled the amount of each constituent to be calculated. The CNS v. was then determined, and on the assumption that the linoleic acid absorbed at one and the linolenic acid at two double bonds, practically the same amount of each constituent was obtained by calculation as before.

We wish to make two criticisms of this experiment. (*a*) In the presence of large amounts of highly unsaturated acids it seems to us very doubtful if the saturated acids or the lithium oleate would be quantitatively precipitated under these conditions. If saturated acid or oleic acid still remained in the linoleic-linolenic mixture, then it is clear that the absorption coefficient used for one or both of these acids must be faulty.

(*b*) Assuming that these two acids do, as Kaufmann and Keller conclude, absorb at one and two double bonds respectively, this relationship may only hold for the particular isomerides of these acids with which they were dealing. This criticism, based on the large number of possible isomerides of both linoleic and linolenic acids, has already been put forward by Van der Veen [1931].

In the present research it has been shown quite clearly that the cocksfoot fatty acids contain large amounts of β-linoleic and β-linolenic acids. As has been stated above, these may both be mixtures of many isomerides. It is possible that isomerisation has taken place during bromination, as Van der Veen suggests, but the very low yield of sativic acid which we obtained by Haworth's method of oxidation, compared with that which the latter himself obtained from linoleic acid isolated from poppy seed and linseed oils, suggests that these isomerides were in large part already present in the original mixed fatty acids.

If we accept Kaufmann and Keller's supposition that these β-linoleic and β-linolenic acids absorb thiocyanogen at one and two double bonds respectively, then the cocksfoot fatty acids must have contained 8·9 % of oleic or some other one double bond acid. In the case of the ryegrass fatty acid we have applied Moore's lithium soap method to isolate any oleic acid (p. 230).

None was found, yet in this case the thiocyanometric method suggested 17·6 % of oleic acid.

We do not believe that our present meagre knowledge of the chemistry of the β-linoleic and β-linolenic acids is sufficient to warrant Kaufmann and Keller's assumption that the thiocyanometric method of analysis can be applied to all mixtures of fatty acids in which they are present. If we assume, for purposes of illustration, that no oleic or other one double bond acid is present in the unsaturated fraction from cocksfoot (Batch P), then the amounts of total linoleic and linolenic acids can be calculated from the I.V. alone, as has already been done in computing the composition given in Table I. The total linoleic acid is 59·5 % and the total linolenic acid is 28·5 %. Using Kaufmann and Keller's equations it is then possible to calculate the thiocyanogen absorption coefficient of either (*a*) the total linolenic acid, or (*b*) the total linoleic acid.

(*a*) The coefficient is 2·4. If the α-linolenic acid (21 %) has a coefficient of 2, then that of the β-linolenic acid (7·5 %) must be 3·5, an impossible value.

(*b*) The coefficient is 1·18. If the α-linoleic acid (38·5 %) has a coefficient of 1, then that of the β-linoleic acid must be 1·5, *i.e.* one-half of the total isomerides grouped together under this name must have a coefficient of 1 and the other half of 2.

We do not suggest that the present results provide definite evidence that certain isomeric octadecadienoic acids absorb thiocyanogen at both their double bonds because the amount of oleic or other one double bond unsaturated acid present in the mixed acids must remain uncertain.

We think, however, that our analytical methods prove that oleic acid cannot be present in cocksfoot and ryegrass glyceride fatty acids to the extent found by the thiocyanometric method. It is not to be inferred from this statement that we query the validity of this method when used by Kaufmann and Keller to determine the composition of mixed seed fatty acids. These acids, especially the less unsaturated acids, have been the subject of much research, and their constitution is known with a fair degree of certainty. But this is not so in the present case. Unlike the seed glycerides the leaf glycerides are not reserve material but are an integral part of the protoplasm of physiologically active cells; and, in the absence of confirming evidence, we have no more right to assume that the highly unsaturated acids derived from them have the same constitution as those derived from the seed glycerides than we have to assume, for instance, that the unsaturated acids of the liver are the same as those of adipose tissue. The discrepancy between the thiocyanometric and the other methods of analysis may be due to the presence of an unknown octadecenoic acid, but equally well to an unknown octadecadienoic or octadecatrienoic acid. Until further evidence for the constitution of the leaf glyceride fatty acids is available, therefore, we consider that the thiocyanometric method of analysis is unreliable.

To summarise, the results of the present research show that the cocksfoot glycerides contain palmitic, stearic, α-linoleic (9 : 12-octadecadienoic) and

α-linolenic acids. Very small amounts of higher saturated acids were found, but these were probably derived from the small amount of wax that was not removed from the ethereal solution of the glycerides by precipitation with acetone.

The glyceride fatty acids of ryegrass.

73 kg. of fresh grass (9·2 kg. dry weight) gave 415 g. of ether extract, from which were obtained 156 g. of mixed fatty acids. The yield was 37·5 % of the ether extract or 1·7 % of the dry weight of the grass. The acids had an I.V. of 175 and a CNS v. of 115·6.

Separation of saturated acids by the method of Bertram.

10·3 g. were taken for oxidation. The saturated acids were extracted as before by light petroleum (B.P. 60–80°) and precipitated twice as the magnesium soaps. The final weight was 1·73 g., equivalent to 16·8 % of the original mixed acids. (Found: C, 75·0; H, 12·6 %; mol. wt. 266. $C_{16}H_{32}O_2$ requires C, 74·9; H, 12·6 %; mol. wt. 256.) The I.V. was 1·6.

Separation of the saturated and unsaturated acids by the method of Twitchell.

10·3 g. of mixed acids treated with 1·7 g. of lead acetate gave 1·32 g. of solid acids of I.V. 13 and M.P. 51·3°. Correcting for the amount of unsaturated acid present the yield was 11·9 % of the original mixed acids. It will be seen that the result is again about 5 % lower than that given by Bertram's method.

α-Linolenic acid. The unsaturated fraction from Twitchell's method described above was brominated in the usual way. The amount of hexabromostearic acid obtained was 4·5 g., M.P. 180°, corresponding to 1·71 g. of α-linolenic acid. The yield was 16·6 % of the original mixed fatty acids.

Thiocyanometric analysis.

The data given above enabled the percentage of the various acids to be calculated, as shown in Table IV. It will be seen that the method suggests the presence of 17·6 % of oleic acid. This is nearly twice the amount which Kaufmann and Keller were able to remove quite readily from linseed oil by means of the lithium soap method of Moore [1919]. We therefore decided to apply this method in the present case.

Attempted separation of oleic acid as lithium oleate.

20·6 g. of the unsaturated acids obtained by Twitchell's method were dissolved in 51·5 cc. of absolute alcohol and treated with an equal volume of water containing slightly more than the theoretical amount of lithium hydroxide required to convert all the acids to the corresponding soaps. The mixture was allowed to stand overnight in the ice-chest. The yellow precipitate was

filtered off and washed with 50 % alcohol. It was recrystallised from the same solvent, the solution being left overnight in the ice-chest. The soaps thus separated were converted into the corresponding acids. 0·83 g. of semi-solid material was obtained, I.V. 92. On recrystallisation from acetone 0·5 g. yielded 0·18 g. of solid acid and 0·32 g. of liquid acid, I.V. 134. It is clear from this result that the amount of oleic acid isolated must have been less than 1 % of the unsaturated acids taken for analysis.

Oxidation of the unsaturated acids by the method of Hilditch.

As in the experiments with the cocksfoot fatty acids the method of alkaline permanganate oxidation gave no recognisable amount of a dihydroxystearic acid, we decided in the present case to apply the acid oxidation method of Hilditch [1926]. 26·5 g. of unsaturated acids separated by Twitchell's method were dissolved in 100 cc. of glacial acetic acid and treated with 4·4 g. of hydrogen peroxide (100 vol.), dissolved in a further 60 cc. of glacial acetic acid. The mixture was allowed to stand at room temperature for 14 days, when a small crystalline precipitate had settled and the containing liquid was otherwise homogeneous. The acetic acid was then removed by distillation in steam, and the residue saponified for 6 hours with 1 litre of *N* sodium hydroxide. 20 g. of dark solid products were thus obtained. Extraction with light petroleum removed 6·5 g. of yellow semi-solid material. The residue was a dark coloured liquid. This was dissolved in a small volume of ethyl acetate, which was then allowed to stand for some days in the ice-chest. The small crystalline precipitate was filtered off, and well washed with ether. The ethereal washings should have contained the dihydroxystearic acid, M.P. 95°, derived from any oleic acid present in the original unsaturated acids, but none was found. The material insoluble in the ether was recrystallised twice from ethyl acetate. The M.P. was 144° and it was not raised by repeated recrystallisation. It was a tetrahydroxystearic acid. (Found: C, 62·0; H, 10·5. $C_{18}H_{36}O_6$ requires C, 62·0; H, 10·4 %.) In the case of the ryegrass fatty acid therefore we are again unable to show the presence of any oleic acid.

Identification of the saturated acids.

Attention has been called above (p. 223) to the fact that the yield of saturated acids given by Twitchell's method is 5–6 % lower than that given by Bertram's method. This may be due to the presence of saturated acids lower in the series than palmitic acid, as the solubility of the lead soaps in alcohol increases rapidly with decrease in molecular weight. To test this supposition 54 g. of the unsaturated acids given by Twitchell's method were converted into the methyl esters in the usual way and then distilled from a Willstätter flask at a pressure of 0·6 mm. Two small fractions only were collected. The first weighed 1·35 g. (B.P. 130°, mol. wt. 244, I.V. 138) and the second 1·83 g. (B.P. 130–150°, mol. wt. 255, I.V. 167). As methyl myristate boils below 100°

Table V. *Fractionation of the saturated fatty acids from ryegrass.*

Esters				Acids						
Fraction	B.P. 0·03 mm.	Weight g.	M.P. (approx.)	Weight g.	Crystalline fractions from acetone	Recrystallised from acetone (A), or from pyridine (P)	M.P.	C %	H %	mol. wt. (titration)
1	116°	1·32	20°	1·3	1st crop 0·62 g.	Once from A	59·5–61°	74·9	12·8	259
						Four times from A	61–61·5°	75·3	12·9	—
					2nd crop 0·3 g.	Once from A	54–55·5°	75·2	12·8	—
2	118°	2·34	22°	2·2	1st crop 1·3 g.	Once from A	60·5–61·5°	—	—	—
						Five times from A	61·8–62·2°	75·2	12·8	259
					2nd crop 0·6 g.	Once from A	60·5–61°	75·2	13·0	263
3	122°	2·63	22°	2·5	1st crop 1·5 g.	Once from A	60·5–61·3°	75·2	12·8	260
					2nd crop 0·6 g.	Once from A	58·5–59°	75·2	12·7	267
4	125–130°	2·89	23–24°	2·7	1st crop 1·7 g.	Once from A	54–56°	74·9	13·0	267
						Four times from A	52–54°	—	—	—
					2nd crop 0·7 g.	Once from A	55–57·5°	75·3	12·7	273
Residue	Not distilled	1·97	31–36°	1·6	1st crop 0·7 g.	Once from A	59·5–60·5°	—	—	—
						Once from P, twice from A	71·5–72·5°	78·2	13·3	353
					2nd crop 0·6 g.	Once from A	50–51·5°	75·8	12·8	282
Ethyl myristate			10·5–11·5°	—	—	Palmitic acid requires	63°	74·9	12·6	256
Ethyl palmitate			25°	—	—	Stearic acid ,,	70°	76·0	12·8	284
Ethyl stearate			34–35°	—	—	"Cerotic acid" ,,	80–82°	78·7	13·2	396

at this pressure it is clear from the boiling-points and I.V. recorded that no appreciable amount of shorter-chain saturated fatty acids was present in this unsaturated fraction. 10·8 g. of saturated acids separated by the Twitchell method were then converted into the ethyl esters in the usual way and distilled at 0·03 mm. The fractions were saponified and the resulting acids crystallised from acetone. Two crops were collected in each case, both of which were then further treated, as in the case of the corresponding acid fractions from cocksfoot. The results are recorded in Table V and can again be interpreted with the aid of the data given in Table III. The first three fractions are nearly pure palmitic acid; Fraction 4 is a mixture of palmitic and stearic acids with a small amount of a higher acid such as eicosanic acid, while the residue is a mixture of longer-chain fatty acids corresponding to "cerotic acid." The approximate analysis—palmitic acid, 70 %; stearic acid, 20 %; "cerotic acid," 10 %—is similar to that of the saturated acid fraction from cocksfoot.

Summary.

The glyceride fatty acids of two forage grasses, cocksfoot and perennial ryegrass, have been investigated in some detail.

The mixed acids are highly unsaturated, and contain a relatively low proportion of saturated acids.

The saturated acids are palmitic and stearic acids, together with a small amount of mixed higher acids similar to "cerotic acid," which may have been derived from wax esters.

The presence of α-linolenic acid was proved by bromination and oxidation, and of α-linoleic acid by oxidation.

Thiocyanometric analysis suggests the presence of oleic or an isomeric octadecenoic acid, but no confirmatory evidence could be obtained by oxidation experiments.

The thiocyanometric method of analysis is discussed in some detail, and the conclusion drawn that it may give misleading results in the case of mixed fatty acids containing large amounts of the so-called β-linoleic and β-linolenic acids. The mixed fatty acids from grasses are of this type.

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XXVII. THE ISOLATION OF ASPARAGINE FROM AN ENZYMIC DIGEST OF EDESTIN.

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Introduction.

One of the most important corollaries of the peptide hypothesis of protein structure is that the dicarboxylic amino-acids exist in the protein molecule, partly or wholly, in the form of their amides. The correctness of this view, however, has never been directly demonstrated by the actual isolation of an acid amide from among the decomposition products of a protein. This is partly due to the fact that the method for the study of protein structure used, almost to the exclusion of all others, *viz.* that of acid hydrolysis, would break down any such acid amides into ammonia and the corresponding amino-acids. It was, in fact, through the observations of Nasse [1872–74] on the production of ammonia during the hydrolysis of proteins by acids and alkalis that the amide hypothesis originated. Fleurent [1893], who observed that large amounts of glutamic acid and ammonia were obtained by the hydrolysis of gliadin, was the first to suggest a possible connection between the ammonia and the dicarboxylic acids.

These views attracted little attention till the development of the peptide hypothesis in 1902, when it was seen that they formed an excellent complement to the theories of Fischer and Hofmeister on the structure of protein, and they finally gained wide acceptance from the work of Osborne *et al.* [1906, 1908] who based their evidence on the apparent equality between the amount of ammonia and of total dicarboxylic acids that could be obtained from a large number of proteins. That most proteins on hydrolysis gave large amounts of glutamic acid and only very small amounts of aspartic acid, so that the amide-N might have its origin in the extremely labile glutamine (capable of being decomposed into ammonia and glutamic acid by boiling with water) and not in the more stable asparagine with which comparison was always made, was a possibility which seems to have been overlooked until Thierfelder and Cramm [1919] drew attention to it. These workers were however able to show that in synthetic dipeptides of glutamine (*viz.* glycyl-, alanyl- and leucyl-glutamine) the amide group was comparable in stability with that of asparagine, and further that the rate of ammonia liberation on acid hydrolysis was closely parallel with that from gliadin.

Developments in the methods of isolating the dicarboxylic acids [Foreman, 1914] and the discovery of β-hydroxyglutamic acid [Dakin, 1918] have lessened the force of Osborne's original argument. According to recent analyses [Damodaran, 1931], in most proteins the dicarboxylic acids are considerably in excess of the ammonia liberated. However, recent measurements of the base-binding capacity of proteins require that there should be such an excess of carboxyl groups in the protein molecule [Cohn, 1925].

If therefore the validity of the peptide hypothesis be admitted, the results of the investigations mentioned above, taken as a whole, do provide strong presumptive evidence for the existence of dicarboxylic acid amides in proteins.

In view of the apparent inadequacy of the peptide theory to explain certain characteristics of proteins many alternative modes of combination of the amino-acids in the protein molecule have been suggested. All these hypotheses postulate the existence in the protein molecule not only of straight polypeptide chains but also of various cyclic structures. In contrast with the peptide theory, none of these hypotheses implies any inherent necessity for the dicarboxylic acids to be present in the form of their amides.

When we turn from the hydrolyses of proteins by acids to those by enzymic action, the results are far less decisive or consistent. Luck [1924] isolated from the tryptic digest of caseinogen a "trypsin-resistant" body which contained about one-third of the total amide-N. His evidence, however, that the ammonia was bound as amide was not very conclusive. Hunter [1925] obtained evidence from caseinogen which suggested that in the early stages of tryptic digestion about one-quarter of the amide-N was present in the form of free acid amides; but no evidence could be found for the liberation of amides during the tryptic digestion of a proteose derived from gliadin, although the amide-N of the latter was more than a quarter of the total N.

Recently, Clementi [1931] has advanced a hypothesis, based upon observations on the deamidation of proteins by enzymes not possessing asparaginase activity, that the ammonia comes not from asparagine but from an amide of the type $R_1.CO.NH.CO.R_2$. He has however overlooked the fact that such a possible precursor of ammonia was discussed by Andersen and Roed-Müller [1915] and rejected by them because compounds of this type were found to be resistant to strong acid hydrolysis.

The aim of the present research was to prove the validity or otherwise of the amide hypothesis by an attempt at actual isolation of an acid amide from the enzymic digest of a protein. If the glutamic and aspartic acids in proteins exist, in part, at least, in the form of their amides, then enzymic digestion, carried sufficiently far and under conditions so regulated as to preclude the hydrolysis of these amides, should make possible the isolation of either or both of these substances from the products of digestion.

That a far-reaching hydrolysis of proteins could be effected by the action, in succession, of pepsin, trypsin and erepsin for comparatively short periods was shown by the work of Frankel [1916]. He found that, in general, 90 % of

the total amino-acids of the proteins investigated were set free by the combined action of the three enzymes, about 16 % of the splitting being due to pepsin, about 50–55 % to trypsin and the remainder to erepsin. The action of pepsin was practically complete in about 3 days, that of trypsin in 5–7 days and of erepsin in 4–5 days.

There were, however, few reliable data available on the liberation of ammonia during the course of these digestions, except in the case of trypsin. The liberation of ammonia during tryptic action was studied by Hunter and Smith [1925] who concluded that this was not, strictly speaking, a function of trypsin at all. The data collected by Henriques and Gjaldbäck [1911, 1912] on the deamidising action of pepsin were of little relevance to the present work as the prolonged digestion of several months was quite uncalled for in view of Frankel's work. A great deal of confusion exists in the older literature on the deamidising action of erepsin. The evidence is extremely conflicting in nature as the experiments were carried out mostly with tissue preparations which have been since shown to contain more than one enzyme.

It was envisaged from the first that yeast dipeptidase, the specificity relations of which are much more sharply defined and much better known than those of intestinal erepsin, could possibly be used as a substitute for the latter in the final stages of digestion. The extensive work of the Willstätter school has established the following facts [Grassmann, 1928].

(*a*) Four proteolytic enzymes—proteinase, polypeptidase, dipeptidase and asparaginase, can be separated from yeast autolysates. The nature of the enzymes present in the autolysate is conditioned by three factors, *viz.* the cell-poison used for plasmolysing the yeast, the reaction at which autolysis is allowed to take place, and the duration of autolysis [Willstätter and Grassmann, 1926].

(*b*) The yeast dipeptidase is only capable of splitting such dipeptides as have a free amino-group and also a free carboxyl group in the α-position with respect to the peptide linkage. The polypeptidase attacks only those peptide linkages which have a free amino- but no free carboxyl group adjacent to them; that is to say, it splits polypeptides and tripeptides, as also dipeptides in which the terminal carboxyl has been substituted, *e.g.* by an alcohol or amide group. Yeast asparaginase is inactive towards any derivative of asparagine in which the amino-group which is in the β-position with respect to the amide group, has been substituted.

The importance of these facts for the isolation of asparagine from an enzymic digest is obvious. Yeast polypeptidase would act on the tri- and higher peptides at the peptide linkage nearest to the free amino-group, hydrolysing them to the dipeptide stage. As the peptide linkage is a substituted amide, the enzyme would also act on the amide group of monoamino-acid amides of the type $R.CH(NH_2).CO.NH_2$ (*e.g.* glycine-amide, leucine-amide) liberating ammonia and the corresponding amino-acid; and on the peptide group of peptide amides of the type $R_1.CH(NH_2).CO.NH.CH(R_2).CO.NH_2$ (*e.g.* glycylleucine-

amide), but would have no action on the amide group in asparagine, $HO_2C.CH_2.CH(NH_2).CO.NH_2$, or on the peptide or amide groups in asparagine dipeptides of the type $R.CH(NH_2).CO.NH.CH(CH_2.CO.NH_2).CO_2H$. A dipeptide containing asparagine would on the other hand be attacked by dipeptidase, the point of attack being however only the peptide linkage. (This holds good whether the asparagine is linked to the rest of the molecule through the carboxyl or the amino-group.) The asparagine thus liberated from peptide combination would remain intact, as only asparaginase is capable of acting on the amide group of asparagine.

In the choice of substrate the main consideration was the selection of a protein with a high aspartic acid content, as the extreme instability of glutamine made it probable that this substance, even if present originally in the protein molecule, would be largely decomposed either during digestion or during the subsequent fractionation. Among the well-defined proteins with a fairly high dicarboxylic acid content edestin has a higher proportion of aspartic to glutamic acid than others, the latest analysis [Jones and Moeller, 1928] showing 10·2 % of aspartic acid and 19·2 % of glutamic acid.

A large number of preliminary experiments were carried out to determine the optimal conditions for obtaining an enzymic digest of edestin suitable for the isolation of asparagine, that is, a digest in which a maximal number of peptide bonds had been split with a minimal liberation of ammonia. With pepsin and trypsin (used in succession) it was soon apparent that the rate of liberation of amino-groups depended on the activity of the samples of enzyme used, while the amount of ammonia present seemed to be independent of enzymic activity and was largely a function of the duration of digestion. Thus, for the same percentage of peptide bonds split, an active preparation of either enzyme liberated a smaller proportion of ammonia than a less active one. With active preparations of both pepsin and trypsin almost maximal liberation of amino-N (about 16–20 % of the total amino-N for the former and a further 45–50 % for the latter) was attained in 3–4 days. Further small increases in amino-N could be obtained by allowing the digestion to continue, but this was found to be inadvisable on account of the loss of amide-N. Thus, in a peptic digest, at the end of 3 days 18·2 % of the amino-N and 5·8 % of the amide-N had been set free. In another digest, which was allowed to continue for 7 days, the amino-N was not much higher (21·05 %), while the free ammonia had increased to 10·6 %. Similar results were obtained with trypsin. In some of the earlier experiments in which the action of pepsin and trypsin was followed by that of intestinal erepsin, the increase in the free amino-N was accompanied by a considerable liberation of ammonia. It is not, however, intended to conclude from this that intestinal erepsin has a definite deamidising action, because in the experiments mentioned above toluene was used as antiseptic, and it is quite conceivable that this was insufficient to exclude completely the activity of intestinal bacteria. Using yeast dipeptidase, in place of erepsin, 93 % of peptide splitting was attained with liberation of but small amounts

of ammonia. Asparagine was eventually isolated from a digest obtained by the successive action of pepsin, trypsin and yeast dipeptidase; over 90 % of the peptide bonds had been split, whereas only about 20 % of the amide-N was present as free ammonia.

Experimental.

Analysis of the edestin. The edestin was prepared from hemp seed in the usual way. No organic solvents were used in the drying, so that it had not undergone any denaturation. It dissolved readily in 10 % sodium chloride solution, and went into solution in 0·1 *N* HCl in the course of about one hour. It contained 10 % moisture; N, moisture-free, was 18·06 %. After hydrolysis with 20 % HCl for 18 hours the amide-N was 10·1 % and the amino-N, after removal of ammonia, 61·6 % of the total N.

Enzyme preparations. Commercial preparations (B.D.H.) of pepsin and trypsin were used throughout. Since the extent of amide splitting appears to depend wholly or in part on the digestion period it is of great importance to use the most active preparations available; those of pepsin and trypsin which attain their maximal amino-N liberation in 72–96 hours were found satisfactory.

The yeast dipeptidase used in the final stage of the digestion was prepared by the method of Willstätter and Grassmann [1926], with the difference that aqueous sodium carbonate instead of ammonia was used to maintain neutrality. 300 g. of pressed beer yeast, after being washed with water and centrifuged, were plasmolysed with 15 cc. of ethyl acetate, diluted to 300 cc. with water, and kept neutral by the addition from time to time of small amounts of a 15 % solution of sodium carbonate. After standing for 1·5 hours the mixture was centrifuged, the residue taken up in 600 cc. of water, and allowed to autolyse for 16 hours at room temperature, the reaction being maintained at p_H 7 by addition of sodium carbonate solution as required. After centrifuging, the clear supernatant liquid contained the dipeptidase, and was found by experiment to be free from asparaginase. According to Willstätter and Grassmann [1926] it probably contained a little polypeptidase, but this was an advantage and no further purification was attempted.

The crude erepsin and also the trypsin-free erepsin used in some of the earlier experiments were obtained from the duodenum of the pig by the methods of Willstätter and Waldschmidt-Leitz.

The *liquor pancreaticus* used in one experiment was the patented preparation of a well-known commercial firm.

Methods of analysis for ammonia-N, amide-N and amino-N.

The ammonia liberated during digestion was determined by the micro-method described by Parnas and Heller [1924] for the estimation of ammonia in blood. The digests were made alkaline with sodium carbonate solution and distilled *in vacuo* with steam into *N*/70 acid till 5 cc. of liquid had condensed

in the receiver. This treatment does not cause any appreciable decomposition of asparagine or glutamine.

Amide-N (after complete hydrolysis with 20 % HCl for 18 hours) was estimated in the Parnas-Wagner micro-Kjeldahl apparatus.

It was found convenient to follow the digestion with each enzyme preparation, to the stage at which it became practically complete, by titration in alcoholic solution [Willstätter, 1921] of the carboxyl groups liberated and then to determine the final amino-N by Van Slyke's method. Comparison could then be made with the total amino-N given by the edestin after complete hydrolysis with 20 % HCl for 18 hours and removal of ammonia. It is necessary here to emphasise that the percentage amino-splitting quoted later in the Tables has no absolute value, because the digests probably contain glutamine, which gives nearly twice the theoretical amount of amino-N by Van Slyke's method with nitrous acid [Chibnall and Westall, 1932].

Glutamine amide-N was estimated by the method of Chibnall and Westall [1932].

Preliminary digestion experiments.

The preliminary experiments to determine the optimal conditions for the isolation of asparagine, *i.e.* of maximum production of amino-groups with minimum production of ammonia, were carried out in the following way.

Table I. *Showing the digestion of edestin by various enzyme preparations.*

	Digest	Enzyme	Duration of digestion (days)	% of total splitting	
				Amino-N	Amide-N
I.	(*a*) Edestin 5 g. Pepsin 0·2 g. *N*/20 HCl 250 cc.	Pepsin followed by	6	21·05	10·6
	(*b*) 125 cc. of (*a*) 10 cc. Na_2CO_3. 15 cc. *Liquor pancreaticus*	*Liquor pancreaticus*	14	57·8	45·9
II.	(*a*) Edestin 5 g. Pepsin 0·2 g. *N*/20 HCl 250 cc.	Pepsin followed by	3	18·2	5·8
	(*b*) 200 cc. of (*a*). 10 cc. of 15 % Na_2CO_3 10 cc. of 5 % trypsin 5 cc. water	Trypsin followed by	3	55·9	13·3
	(*c*) 100 cc. of (*b*) 25 cc. erepsin solution	(i) Erepsin (purified) (ii) Erepsin (crude)	5 5	90·9 90·1	50·3 62·8
III.	(*a*) Edestin 20 g. Pepsin 1 g. *N*/20 HCl 1000 cc.	Pepsin followed by	3	18·1	6·2
	(*b*) 750 cc. (*a*) Trypsin 0·7 g. 65 cc. 5/7 *N* NaOH	Trypsin followed by	4	66·7	16·25
	(*c*) 500 cc. tryptic digest 50 cc. yeast dipeptidase 50 cc. 0·1 *N* HCl	Yeast dipeptidase	5	91·9	22·4

5 g. edestin were dissolved in 125 cc. of 0·1 *N* HCl. About 1 hour was normally required for complete solution at 37°. 0·2 or 0·25 g. of pepsin dissolved in water was added, together with sufficient water to make the volume at 37° up to 250 cc. 5 cc. portions were removed periodically for the determination of amino-N and of ammonia-N. When peptic digestion had reached completion, 200 cc. of the solution were removed, brought to p_H 8·5 by the addition of *N* NaOH, and 10 cc. of a solution containing 0·2 g. trypsin added. 0·5 % by volume of tricresol was used as antiseptic. At the end of the tryptic digestion a solution of erepsin or yeast dipeptidase (1/10 vol.) was added; in the latter case the reaction of the digest was adjusted to p_H 7 by the addition of 0·1 *N* HCl. The results of some typical digests are given in Table I.

It will be seen that the enzymes pepsin, trypsin and yeast dipeptidase used in succession are the most favourable for the present purpose.

Preparation of a large-scale digest.

The large-scale digest for the fractionation of the amino-acids and amides was prepared in the following way. 70 g. of edestin (63 g. of moisture-free protein) were dispersed in 1750 cc. of 0·1 *N* HCl and 3·5 g. of very active pepsin dissolved in an equal volume of water added. The digest was incubated at 37° and the hydrolysis followed by titrating 10 cc. portions in alcoholic solution against 0·1 *N* alcoholic potassium hydroxide. At the end of 90 hours the maximum amount of free amino-N was attained. 3430 cc. of the digest were next brought to p_H 8·6 by the addition of 220 cc. of *N* NaOH. 3·5 g. of an active preparation of trypsin dissolved in 25 cc. of water were added, together with 15 cc. of tricresol. 10 cc. samples were withdrawn at intervals and titrated as before. Tryptic digestion was complete at the end of 4 days. The remainder of the solution (3630 cc.) was brought to p_H 7 by the addition of 30 cc. of *N* HCl and 350 cc. of yeast dipeptidase were added. At the end of 4 days the liberation of amino-groups had almost ceased.

The extent of the protein hydrolysis is shown in Table II.

Table II. *Showing the extent of amino- and amide-hydrolysis in the large-scale digest of edestin.*

Enzyme	Duration of digestion	Splitting %	
		Amino-N	Amide-N
Pepsin	90 hours	20·6	6·8
Trypsin	4 days	59·8	15·7
Yeast dipeptidase	4 days	92·3	22·2

Fractionation of the large-scale digest.

(Analyses of the digest at various stages of fractionation are given in Table III. The appropriate analysis letter is given in the text.)

3800 cc. of the digest were brought to a slightly acid reaction (p_H 6·5) to avoid possible loss of free ammonia, concentrated *in vacuo* at 40° and the rather turbid liquid thus obtained made up to 500 cc. (A).

The solution was left overnight in the ice-chest, when a small amount of solid material separated. This was filtered off, the filtrate and washings were made alkaline to p_H 10 with baryta, the precipitate obtained was removed by centrifuging, and the solution and washings were concentrated *in vacuo* to about a third of the original volume to remove ammonia. A small amount of solid material separated during the concentration and was filtered off. The filtrate was made up to 500 cc. (B).

Removal of basic substances and peptides. 495 cc. of this filtrate were cooled to 0°, treated with sulphuric acid to remove barium, the barium sulphate was centrifuged off and washed repeatedly with water at room temperature. The filtrate and washings (about 800 cc.) were cooled to 0° and kept at this temperature while sulphuric acid (to 5 % by volume) was added. The solution was then treated with about 1000 cc. of phosphotungstic acid (20 % in 5 % sulphuric acid) till precipitation was complete. The precipitate was removed by centrifuging and washed with some of the phosphotungstic acid solution. Phosphotungstic acid was removed from the clarified digest by treating with a thin cream of baryta to strong alkalinity. The barium phosphotungstate was filtered off and the filtrate brought to p_H 7 with sulphuric acid. The barium sulphate was removed by filtration and the filtrate evaporated *in vacuo* to about 300 cc. To ensure complete removal of phosphotungstic acid the solution was once more made strongly alkaline with baryta. The slight turbidity was removed by centrifuging and the clear solution made up to 500 cc. for analysis.

Removal of free dicarboxylic acids. 495 cc. of this solution were again brought to p_H 7, evaporated *in vacuo* to 300 cc., made strongly alkaline with baryta and treated with 3 volumes of alcohol. The precipitated barium dicarboxylates were filtered and washed with 95 % alcohol. The filtrate and washings were brought to p_H 7 with sulphuric acid, the barium sulphate removed and the solution and washings concentrated *in vacuo* and made up to 500 cc. (D).

Separation of tyrosine and other sparingly soluble material. 495 cc. of the above solution (containing 3·653 g. N) were concentrated *in vacuo* at 40° to 200 cc. when crystalline material began to separate. After standing overnight the crystals (0·73 g.) were filtered off and recrystallised. The material was tyrosine. (Found: N, 7·78. $C_9H_{11}O_3N$ requires N, 7·74 %.) The solution was again concentrated *in vacuo* to 150 cc. and then allowed to stand in the refrigerator. A further amount of crystalline material separated. It was not identified, but was shown by complete hydrolysis to contain no amide-N. The mother-liquor and washings were made up to 250 cc. and 25 cc. withdrawn for analysis (E); dicarboxylic acid-N was determined by the method of Damodaran [1931].

Removal of hydrochloric acid. It was thought advisable at this stage to free the solution from hydrochloric acid, which had been present since the initial peptic digestion.

The solution was treated with finely powdered silver sulphate, added in small quantities at a time with vigorous stirring, till the precipitation of silver

Table III. *Fractionation ... destin digest by means of various reagents.*

Fraction	Treatment	Total N (g).	% total N of the fraction: Free ammonia-N	Amino-N	Amide-N (including free ammonia-N)	Other determinations made	Total N in solution used for next operation (g.)	Expressed as percentage of total N in original digest, allowance being made for aliquots withdrawn: Total N	Free ammonia-N	Amino-N	Amide-N (including free ammonia-N)	Other determinations made
(A)	Original digest after concentration	11·64	2·3	59·6	10·8	—	11·52	100	2·3	59·6	10·8	—
(B)	After precipitation with baryta and removal of ammonia	9·01	0·24	59·2	9·5	—	8·92	78·18	0·19	46·3	7·45	—
(C)	After precipitation with phosphotungstic acid and removal of reagents	4·30	—	81·3	12·7	—	4·26	37·70	—	30·6	4·8	—
(D)	After precipitation of dicarboxylic acids	3·69	—	81·7	—	Amino-N on complete hydrolysis: 88·4 Peptide-N : 6·7	3·65	32·68	—	26·7	—	Peptide-N 2·2
(E)	After crystallisation of tyrosine and other insoluble material	3·35	—	—	12·0	Dicarboxylic acid-N on complete hydrolysis: 20·7	3·02	29·96	—		3·6	Dicarboxylic acid-N 6·2
(F)	After removal of chlorides with silver sulphate	3·01	0·81	—	12·0	Glutamine amide-N 6·0	—	29·92	0·24	—	—	Glutamine amide-N 1·7

Table IV. *Analysis of the various crystal fractions.*

Crystal fraction	Volume of solution (cc.)	Nature of material crystallising out	Weight (g.)	N (moisture-free %)	N (ash- and moisture-free %)	Amide-N (% of total N)
1	125	Thick, long prisms, mixed with powdery material	5·7	3·99	7·1	1·1
2	100	Same appearance as 1	8·6	5·57	9·84	1·1
3	60–50	White powder	1·74	15·3	16·81	33·3
4	35–30	White powder	0·28	10·0	—	7·0
5	25	White powder, extremely hygroscopic, sticky even after drying at 100° over P_2O_5 *in vacuo*	1·2	11·09	—	7·8
6	20	Buttery solid formed on long standing	*ca.* 2·0	11·64	—	10·4
7	Obtained from the thick syrupy mother-liquor by precipitation with alcohol	—	—	12·1	—	14·58

chloride was complete. The excess of silver was then removed by hydrogen sulphide and the solution freed from the latter by aeration. Sulphuric acid was then removed by adding a saturated solution of barium hydroxide to p_H 7. The barium sulphate was filtered off and thoroughly washed. The filtrate and washings were then concentrated *in vacuo* and made up to 500 cc. (F).

Isolation of asparagine.

The solution was next concentrated *in vacuo* to about 125 cc., when crystalline material began to separate. From this stage onward successive crops of crystals were obtained by slow concentration of the solution in a vacuum desiccator over sulphuric acid. When separation of crystals began the solution was removed from the desiccator and left in the refrigerator, usually for 2–3 days. At the end of this time the crystals were filtered off and the mother-liquor left for another day or two in the refrigerator to see if crystallisation of the particular crop was complete. The solution was then further concentrated till it again showed signs of crystallisation when it was again transferred to the refrigerator. Each fraction as it separated was filtered with suction and washed with a little ice-cold water, dried *in vacuo* over H_2SO_4 and analysed for total N and amide-N.

The data for the different fractions are given in Table IV.

It will be readily seen that the most promising fraction from the point of view of the present research was 3, which yielded exactly one-third of its total N as amide-N on hydrolysis. The amino-N before hydrolysis was 65 % and after hydrolysis 68 % of the total N.

From these results it was clear that the substance was a mixture of an amino-acid with an amino-acid amide. The distant possibility that it was a peptide of glutamine giving an abnormal amino-N value similar to that given by glutamine itself was excluded by the results of analysis for carbon and hydrogen.

The material readily recrystallised from a little warm water in numerous small prisms, and on further recrystallisation was obtained in the transparent rhombic prisms typical of asparagine. These were filtered off and washed successively with 95 % alcohol, absolute alcohol and ether. 0·96 g. of asparagine was thus obtained (equivalent to 1·11 g. allowing for the aliquots removed for the various analyses). It gave the pale purple biuret reaction characteristic of a β-amide such as asparagine. The air-dried material contained 19·0 % N; calculated for $C_4H_8O_3N_2$, H_2O, 18·65 %. After drying *in vacuo* at 100° over phosphorus pentoxide the anhydrous material contained 21·1 % N. $C_4H_8O_3N_2$ requires 21·2 % N. The amino-N was 50·8 % of the total N. After hydrolysis for 2 hours with *N* hydrochloric acid the amide-N was 50 % of the total N.

From the mother-liquor no further crystals of asparagine could be obtained. The white powdery substance which separated on concentrating the solution

in vacuo had a nitrogen content of 11 %, and on complete hydrolysis the amide-N was 0·6 % of the total N.

The thick syrup after the removal of Fraction 6 showed no tendency to crystallise. It was treated with absolute alcohol till precipitation was complete. The material obtained was extremely hygroscopic and could not be dehydrated even by repeated treatment with acetone. A portion was dried over phosphorus pentoxide *in vacuo* for analysis; N, 12·1 %. The amide-N was 14·6 % and glutamine amide-N 6·0 % of total N.

As it was not found possible to obtain anything crystalline from this syrupy material, and as the amide-N in Fractions IV, V and VI was too small to permit of the isolation of any asparagine or glutamine, all the fractions were dissolved in water, combined and treated with mercuric nitrate solution for the precipitation of the dicarboxylic acid amides. The small precipitate so obtained was decomposed with hydrogen sulphide and, after removal of the latter, the solution was concentrated *in vacuo* to a syrup. No crystalline material was obtained even after standing for some weeks.

Discussion.

No calculations can be made of the exact relation which the weight of asparagine isolated bears to the amount present in the original protein. The total dicarboxylic acid-N in edestin is 15·6 %, made up of 5·8 % aspartic acid-N and 9·85 % of glutamic acid-N, while the amide-N is 10·1 %, so that not more than 64·7 % of the total dicarboxylic acids can be present as amides. We have at present no means of ascertaining the distribution of the ammonia between the two acids, but, assuming arbitrarily that it is equally divided between them, the amount of asparagine ($C_4H_8O_3N_2$, H_2O) in 63 g. of edestin is 4·4 g. The amount of asparagine isolated (1·11 g.) is therefore not unduly small when it is remembered that the yields of amino-acids hitherto obtained from enzymic digests of proteins are generally very much lower than those obtained after acid hydrolysis. The degree of hydrolysis attained with enzymes is far from being complete, even the 90 % splitting calculated from the Van Slyke amino-N representing too high a value on account of the abnormal behaviour of glutamine. As will be seen from Table III baryta and phosphotungstic acid together removed material containing 26·2 % of the total amide-N, so that if this had its origin in asparagine or glutamine both of these amides must still have been present as components of peptides.

A legitimate calculation of the percentage yield of asparagine can, however, be made on the basis of the analytical values for Fraction F (Table III), where the digest consists almost entirely of simple amino-acids. The amide-N in this fraction is 11·2%, of which 6·0 % is shown to be due to glutamine. The maximum possible amount of asparagine amide-N is therefore 5·2 % of the total N or 0·17 g. equivalent to 1·82 g. of asparagine. The 0·96 g. of asparagine isolated therefore represents a yield of 52·7 %.

Glutamine is known to crystallise from solution only with great difficulty in the presence of much foreign material, so that the failure to isolate this amide is not surprising. There seems to be no doubt, however, from the work of Chibnall and Westall [1932], that free glutamine was present in Fraction F. In the present research a protein giving a high yield of aspartic acid on acid hydrolysis was chosen so as to facilitate the isolation of asparagine. To isolate glutamine a protein rich in glutamic acid, and as nearly as possible free from hydroxyglutamic acid, such as gliadin, should be used, and experimental conditions adjusted so as to prevent undue decomposition of this very labile substance at the stage when it is set free from peptide combination. Work on these lines is already in progress in this laboratory.

There is the possibility that some of the amide-N of edestin has its origin in monocarboxylic acid amides such as glycine-amide. As was mentioned in the Introduction such substances are hydrolysed at the amide group by yeast polypeptidase; the yeast dipeptidase used in the present research contained some polypeptidase, so that amides of this type would have been partially or wholly decomposed. But the amount of ammonia set free during this stage of the digestion was only 6·5 % of the amide-N, so that this figure represents about the maximal amount of such amides which could have been present. It should be pointed out, however, that on the peptide hypothesis such amides could only occur at the end of a polypeptide chain.

The possibility also cannot be excluded that a small amount of the ammonia produced on mild acid hydrolysis has its origin in some linkage other than the amide group. Uramino-acids of the type $R.CH(CO_2H).NH.CONH_2$ have been suggested by Lippich [1914] and by Andersen and Roed-Müller [1915] as possible sources of ammonia. Neither of these observers was able to isolate such compounds from protein digests, but indirect evidence, based on the liberation of carbon dioxide during hydrolysis, was produced to show their possible presence. The question of their existence in the protein molecule cannot however be profitably discussed in the present paper, because, as far as is known, such linkages are not attacked by proteolytic enzymes.

Summary.

A practically complete digestion of edestin, with but little splitting of amide-N, has been attained by acting on the protein with pepsin, trypsin and yeast dipeptidase in succession. Complex polypeptides, bases and dicarboxylic acids were removed from the digest by appropriate reagents, and the resulting solution fractionally crystallised. From one of the crystal fractions asparagine was isolated and its presence in the protein molecule thus definitely established. The attempt to crystallise glutamine was unsuccessful but its presence in the digest was demonstrated indirectly by the method of Chibnall and Westall.

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XXVIII. THE SPECIFIC CARBOHYDRATE OF THE TUBERCLE BACILLUS.

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The first experiments on the hydrolysis of the specific carbohydrate from tubercle bacilli [Laidlaw and Dudley, 1925] indicated that only a part of the total hydrolytic products could be accounted for as reducing sugars. Later work by Masucci, McAlpine and Glenn [1930] and Renfrew [1930] has shown that amongst the products acidic substances are formed. The present communication deals with the attempted identification of these acids.

Prolonged acidic hydrolysis led to the exclusive formation of non-acidic, alcohol-soluble substances, but hydrolysis with 2·5 % sulphuric acid for a short time yielded mannose, *d*-arabinose, galactose, and an acidic residue, which, when isolated as the calcium salt, amounted to 30 % of the weight of the carbohydrate. In different experiments the calcium content of this salt varied from 4 to 6 % and attempts to purify it showed it to be heterogeneous; *e.g.* fractional precipitation of an aqueous solution with alcohol yielded a series of salts with calcium contents ranging from 10 to 2·5 %. A similar product was formed by hydrolysis of the carbohydrate with aqueous oxalic acid and longer treatment with *N* sulphuric acid led first to the production of a smaller amount of acid of approximately the same average molecular weight and finally to complete loss of acidic character. In all these repeated hydrolyses of the acidic material mannose is produced almost exclusively of any other reducing sugar.

The carbohydrate used in these experiments was free from nitrogen and sulphur and thus the possibility that the acidic constituents consisted partly of amino-acids or acid sulphuric esters was excluded. The small content of organically combined phosphorus accounts for only a small fraction of the total acid produced.

Although it has not been found possible to identify the acidic constituents, the results so far obtained make it possible to submit a tentative suggestion as to the general type of the structure of the material. It appears to contain a comparatively stable acidic nucleus, consisting of a polymerised glucoside-like compound of mannose and sugar acids, which is less firmly combined with arabinose, mannose and galactose molecules. A part of the acids may consist of substances of the sugar phosphoryl ester type.

It is difficult to obtain evidence as to whether the specific substance consists of a series of carbohydrates of the same type with specific properties, or

whether it contains a small amount of a highly active specific substance mixed with inactive substances of a closely similar type. The first view is considered the more probable since, by fractional precipitation and adsorption, products with very different optical rotations were obtained, which, however, displayed essentially equal specific activities.

Experimental.

Growth of the bacillus.

Cultures of the human strain 100 of the tubercle bacillus were made at 37° on a modified Long's medium devised by Capt. Douglas. It had the following composition: tap-water, 1000 cc.; glycerol, 50 cc.; sodium citrate, 6 g.; potassium dihydrogen phosphate, 2 g.; ammonium chloride, 1 g.; hydrated magnesium sulphate, 0·5 g.; ferrous ammonium citrate, 0·05 g.; glycine, 4 g.; guanidine hydrochloride, 5 cc. of a 2·2 % solution.

Isolation of the carbohydrate.

The original method of Laidlaw and Dudley was modified in order to shorten the process. Moist tubercle bacilli were suspended in sufficient 4 % sodium hydroxide solution (containing 4 % of sodium acetate) to form a very thin paste and heated on the water-bath until most of the material had passed into solution (4–5 hours). After cooling the liquid was rendered acid with acetic acid and the precipitate removed. An excess of copper sulphate was added and the impure salt of tuberculinic acid filtered off. After removal of excess of copper by means of hydrogen sulphide, the liquid was reduced to a small volume at 40° and dialysed in collodion sacs until free from dialysable substances. The liquid was then evaporated to a thin syrup and poured into ten volumes of absolute alcohol. The crude carbohydrate was purified and separated from glycogen as previously described [Laidlaw and Dudley, 1925]. Later experiments showed that the lengthy process of dialysis could be avoided by precipitation of the carbohydrate with basic lead acetate in the presence of ammonia.

After removal of the precipitate produced by acetic acid, the filtrate was treated with an excess of normal lead acetate and the precipitate discarded. Addition of a slight excess of basic lead acetate, after basifying the filtrate with ammonia, produced a heavy precipitate. This was suspended in water and treated with acetic acid until no more material passed into solution. The undissolved material was discarded and the dissolved substances reprecipitated with ammonia. This process was repeated until the material dissolved completely in dilute acetic acid. The lead salts were then decomposed with hydrogen sulphide and the carbohydrate was isolated as before.

Preparations were also made from the medium upon which tubercle bacilli had been grown in the same way. The carbohydrate thus obtained possessed

properties similar to those previously recorded, but although no glycogen could be detected as a contaminant, the optical rotatory power was very variable.

Origin of preparation	$[\alpha]_{5461}$ in water
Autoclaved bacilli	+32·1°
Autolysed bacilli (kept at 40° for several months)	+50·3
Untreated bacilli	+83·1
Old tuberculin (from Long's medium)	+37·3
Long's medium (12 weeks' culture)	+89·1

These samples gave about 4 % of ash upon ignition, but this content could be reduced to below 1 % by repeated precipitation with alcohol from solutions containing hydrochloric acid. The rotatory powers of the samples were not altered by the addition of 2 % of hydrochloric acid. Most samples contained no nitrogen detectable by the Kjeldahl method, but contained about 0·8 % of phosphorus. Samples whose ash-values had been reduced below 1 % were strongly acidic in reaction. The precipitating power against immune serum varied from 1 in 5 millions to 1 in 3 millions. Glycogen was isolated only from the untreated bacilli. The yield of carbohydrate from a 12 weeks-old culture on the modified Long's medium (17,000 cc.) was 8–12 g. from the bacilli and 5–10 g. from the medium. In older cultures more appeared in the medium.

Fractionation of carbohydrate.

Fractional precipitation of a solution of the carbohydrate (0·9 g., $[\alpha]_{5461}$ + 50·3°) in water (4 cc.) with alcohol (12 cc.) yielded a precipitated fraction (0·33 g., $[\alpha]_{5461}$ + 59·8°) and an unprecipitated fraction (0·5 g., $[\alpha]_{5461}$ + 47·3°). Other fractionations gave similar results, as did fractional precipitation by alcohol of a solution of the carbohydrate in formamide. Precipitation of a solution of the carbohydrate (0·2 g., $[\alpha]_{5461}$ + 83·1°, from untreated bacilli) in water (2 cc.), with insufficient saturated basic lead acetate solution to give complete precipitation (1 cc.) and 2*N* ammonium hydroxide (0·5 cc.), followed by recovery of the carbohydrate from the precipitate and the filtrate by decomposition with hydrogen sulphide, yielded fractions with + 91·4° (0·126 g.) and + 71·0° (0·038 g.) respectively.

It was observed that an aqueous solution of the carbohydrate containing lithium, barium, strontium or calcium chloride and just insufficient alcohol to cause precipitation gave a precipitate upon addition of ammonia. This solid contained a large amount of the metal and possessed an alkaline reaction. A solution of carbohydrate (1·0 g., ash content 0·91 %, $[\alpha]_{5461}$ + 89·1°) in water (9 cc.) and ethyl alcohol (8 cc.) containing saturated calcium chloride solution (0·8 cc.), was treated with concentrated aqueous ammonia (1 cc.) and the precipitated solid removed and washed with 75 % alcohol. The washings and filtrate were poured into a large excess of absolute alcohol. The precipitate

(0·22 g.) gave 8·2 % ash, had $[\alpha]_{5461}$ + 82·4° and contained 0·83 % P, whilst the substance (0·7 g.) recovered from the mother-liquors gave 2·3 % ash, had $[\alpha]_{5461}$ + 111·8° and contained 0·45 % P.

Acetylation of the carbohydrate.

The carbohydrate (0·11 g., $[\alpha]_{5461}$ + 52·8°) was warmed with a mixture of pyridine (1 cc.) and acetic anhydride (1 cc.) at 40°. The solid slowly dissolved and after some hours a flocculent precipitate separated. After 24 hours the whole mixture was poured on to ice and the precipitated solid removed (0·108 g.). The product was almost insoluble in water, ether, light petroleum and carbon disulphide but dissolved in alcohol, acetone, chloroform or benzene. Addition of small amounts of water to the alcoholic solution produced an amorphous precipitate. The acetylated product had $[\alpha]_{5461}$ + 42·9° in ethyl alcohol. The carbohydrate was recovered from the acetyl derivative (0·1 g.) by warming at 100° with *N* sodium hydroxide (5 cc.) for 2 hours. The recovered material had $[\alpha]_{5461}$ + 95·4° (prepared from a sample of carbohydrate $[\alpha]_{5461}$ + 91·4°) and possessed essentially the same specific activity.

Adsorption experiments.

Attempts to adsorb the carbohydrate on benzoic acid, α-naphthol and diphenylmethane-2 : 4′-dicarboxylic acid were unsuccessful. Adsorption took place readily, however, on Lloyd's reagent. A solution of the carbohydrate (0·313 g., $[\alpha]_{5461}$ + 84°) in 0·06 *N* hydrochloric acid (30 cc.) was shaken with Lloyd's reagent (4·2 g.) for 2 minutes and filtered. The filtrate was freed from aluminium and dialysed, giving finally 0·14 g. carbohydrate, $[\alpha]_{5461}$ + 69·4°. The adsorbate was eluted with *N*/20 sodium hydroxide (30 cc.), the eluate freed from aluminium and dialysed. From the eluate 0·11 g. carbohydrate was obtained with $[\alpha]_{5461}$ + 94·1°. Both products had very similar precipitating powers towards immune serum.

Hydrolysis experiments.

Preliminary experiments showed that no change in rotatory power or production of reducing sugars was brought about by the action of saliva, malt diastase or taka-diastase. The stomach juices of the snail, which have been used by Karrer to hydrolyse various cellulose derivatives, were equally without action on the specific carbohydrate. 0·1 *N* hydrochloric acid at 40° has almost no action on the carbohydrate. At 100°, however, 1 % sulphuric acid produced the rapid formation of reducing sugars, the reduction value as measured by the Hagedorn-Jensen method reaching a maximum in 2 hours. A very slight fall in reducing power occurred after this period, amounting to a 6 % loss of maximum reducing power after 5½ hours. In order to isolate the hydrolytic products the carbohydrate (2 g.) was heated at 100° for 3 hours in 2·5 % sulphuric acid (100 cc.). The observed optical rotation of the solution fell rapidly from

α + 1·89° to + 0·01° in 2 hours. After 3·5 hours a slight negative rotation was observed (− 0·02°). After quantitative removal of the sulphuric acid with baryta the liquid was digested with an excess of calcium carbonate at 100° until it became permanently neutral to litmus. A separation of the acidic components was effected by evaporation of the neutral filtrate to a small volume followed by precipitation with ten volumes of methyl alcohol. The insoluble material was washed repeatedly to remove adhering sugars.

The alcohol-soluble portion was freed from alcohol, dissolved in water (15 cc.) and treated according to the method of Chargaff and Anderson [1930]. Addition of phenylhydrazine (1·5 g.) dissolved in alcohol (3 cc.) to the liquid resulted in the formation of mannosephenylhydrazone (0·14 g.). After crystallisation from 60 % ethyl alcohol it formed small rhombs and had M.P. 194° (not depressed by admixture of authentic mannosephenylhydrazone). The filtrate from this hydrazone was shaken with freshly distilled benzaldehyde (2 g.), kept for 12 hours, filtered and extracted three times with ether. After evaporation (to 7 cc.) the liquid was treated with α-benzylphenylhydrazine (1·2 g.) in alcohol (2 cc.) and sufficient alcohol to give a homogeneous solution. After keeping for 12 hours the mass of fine needles (0·67 g.) was removed and crystallised from 60 % alcohol. *d*-Arabinosebenzylphenylhydrazone was thus obtained with M.P. 175–176°. The filtrate was then boiled with benzaldehyde (1·2 g.) for 2 hours. After dilution with water (50 cc.) the liquid was filtered, extracted four times with ether, and evaporated to 3 cc. Addition of freshly distilled α-phenylmethylhydrazine (1·5 g.) and sufficient alcohol to give a clear solution produced a small amount of a substance crystallising in elongated rectangular plates (0·04 g.). After crystallisation from aqueous alcohol this substance had M.P. 157° and did not depress the melting-point of pure galactose-α-phenylmethylhydrazone.

The alcohol-insoluble portion (0·6 g.), consisting of calcium salts, gave 3·1 % of ash on ignition, possessed a weak reducing power towards Fehling's solution and yielded a strongly acidic substance after precipitation from dilute hydrochloric acid with alcohol. Another sample of the calcium salt prepared in a similar way gave a reduction value equivalent to 4·9 % glucose by the Hagedorn-Jensen method. All samples of the salt prepared gave a negative naphthoresorcinol reaction for glycuronic acid.

Samples of calcium salt prepared from the carbohydrate in the same way possessed calcium contents varying from 4 to 6 %. Fractional precipitation of the salt (1·5 g.) from aqueous solution (6 cc.) by alcohol yielded a fraction slightly soluble in water (0·1 g.) and three very soluble fractions (0·25, 0·50, and 0·41 g.) which possessed calcium contents 10·03, 5·45, and 2·54 %, respectively. The last three fractions were non-reducing to Fehling's solution. The last two fractions were combined and heated with *N* sulphuric acid at 100° with the object of obtaining a simpler hydrolytic product. Two fractions of calcium salt were isolated (0·044 g., Ca 8·55 %; 0·007 g., Ca 7·28 %), and from the alcohol-soluble hydrolytic products mannosephenylhydrazone (0·15 g.)

was obtained by the action of phenylhydrazine. Only a very small amount of an osazone could be prepared from the mother-liquors of the mannosephenylhydrazone.

Since hydrolysis with sulphuric acid caused considerable darkening, a milder hydrolytic agent was sought and it was found that hydrolysis of the carbohydrate (4·4 g.) with oxalic acid (5 g.) in water (50 cc.) at 100° for 12 hours gave rise to a much larger yield of calcium salt (1·3 g., Ca 6·22 %). Further hydrolysis of this salt with *N* sulphuric acid at 100° resulted in some darkening and loss of much total acidic content. The acid from this hydrolysis, isolated as the barium salt (0·13 g., Ba 19·6 %), possessed almost the same equivalent as the original material. Reducing sugars were produced as in the previous experiment. The barium salt which was extremely soluble in water gave an intense yellow colour with ferric chloride solution and also gave the reaction of Denigès [1909] for glycollic acid. Attempts to isolate calcium glycollate by conversion into the calcium salt and seeding with calcium glycollate were not successful. At this stage it was thought possible that the salt might contain some aldehyde-acid unit of the glycuronic acid type which decomposed when the glucoside linking was broken. An attempt therefore was made to effect hydrolysis and oxidation at the same time with the production of a dicarboxylic acid. Oxidation of a sample of the calcium salt (0·12 g., Ca 6·22 %) with *N* hydrobromic acid (2·5 cc.), containing 0·5 cc. of bromine in 50 cc., in a sealed tube at 102° for 22 hours, resulted in complete loss of acidic value.

Fractionation of the hydrolytic acids was also made by means of lead salts. A sample of carbohydrate (4·4 g.), isolated from tubercle medium, was hydrolysed with oxalic acid as before and the acid products were isolated as the calcium salts (0·68 g., Ca 3·0 %). The calcium ion was quantitatively removed with oxalic acid from a solution of the total salts in water (20 cc.) and the resulting acid divided into two fractions by the addition of alcohol. The precipitated material (0·08 g.) was heated for 10 hours with oxalic acid (1 g.) and water, when small amounts of phosphate ion and reducing sugars were formed. The recovered material, which was free from organically combined phosphorus, gave no precipitate with basic lead acetate in neutral solution; on the addition of ammonia a heavy precipitate was produced. The material unprecipitated by aqueous alcohol in the first separation of the acidic constituents was isolated by evaporation, followed by precipitation from a large volume of alcohol. Evaporation of the alcohol liquors yielded a minute amount of an acidic syrup which gave a strong reaction for glycollic acid but which could not be converted into a crystalline calcium salt. The alcohol-insoluble material (0·4 g.), which was slightly acid to Congo red and non-reducing towards Fehling's solution, was treated with lead acetate in water (8 cc.) and a small amount of mainly inorganic material, containing 0·2 % P, removed. After neutralisation with ammonia, lead acetate was added to the filtrate, the precipitate removed, the mother-liquor made alkaline with ammonia and an excess of basic lead

acetate added. Decomposition of the precipitate from neutral solution with hydrogen sulphide gave a small amount of acidic substance, containing less than 0·2 % of phosphorus, which gave a glycollic acid reaction. The precipitate formed in alkaline solution was similarly decomposed and yielded an acidic gum (0·15 g.) insoluble in alcohol and acid to Congo red (P, 0·78 %; 5·132 mg. required 0·42 cc., 0·0135 *N* sodium hydroxide for neutralisation; equivalent, 905). Attempts to form a crystalline brucine salt were not successful. A barium salt of the acid was prepared by digestion of the acid with an excess of barium carbonate and separated into two fractions by partial precipitation from a small volume of water with ethyl alcohol. The less soluble fraction contained 1·66 % and the more soluble 0·42 % of phosphorus.

Summary.

1. Methods of isolation of the specific carbohydrate from tubercle bacilli and from the medium on which they have been grown are described.

2. Acetylation of the carbohydrate with acetic anhydride and pyridine yields a water-insoluble derivative from which the carbohydrate may be regenerated by alkaline hydrolysis without detectable loss of specific precipitating activity.

3. Hydrolysis of the carbohydrate with acids yields mannose, *d*-arabinose, galactose and a mixture of acids which partially lose their acidic nature and give mannose on prolonged hydrolysis. Evidence of the presence of an acid, possibly glycollic, was obtained, but no glycuronic acid could be detected.

The author is deeply indebted to Capt. S. R. Douglas for his help in supplying the tubercle cultures and for testing the specific precipitating power of the products.

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XXIX. THE RESERVE POLYSACCHARIDE OF THE SEEDS OF FENUGREEK: ITS DIGESTIBILITY AND ITS FATE DURING GERMINATION.

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Introductory.

The seeds of fenugreek (*Trigonella Foenum-Graecum* L.) have been employed, for many centuries, as a drug in Southern Europe, Northern Africa, Asia Minor and India. They are extensively used by many people in Egypt, not only as a food but also as a medicine for different ailments which appear to have no relationship to one another.

The use of the seeds as a food can be seen among the field labourers who constitute the major part of the population and live almost entirely on maize bread to which a small percentage of the ground fenugreek seeds is added. They are also utilised as a food by women in order to give plumpness to their forms.

For medicinal purposes, the seeds are employed as a stomachic, also as a laxative in the form of powder or a decoction. They are given to women after parturition in the form of a decoction sweetened with sugar and form one of the chief ingredients of pills or powders which are administered for the treatment of diabetes mellitus. For some of the purposes mentioned the seeds are used in the germinated form.

Apart from their nutritive value and bitterness which may account for their stomachic properties, they are not known to contain any active principle of therapeutic value. On the other hand, it is known that mucilages act as emollients provided they are not attacked in the alimentary canal and since fenugreek seeds contain a high percentage of a polysaccharide, highly mucilaginous in character, this may explain their use as a laxative. It may also account for their employment in anti-diabetic preparations, since diabetics generally suffer from constipation and any improvement which follows their use may be indirectly brought about by relieving this symptom. The mucilage, as will be seen later, does not influence the blood-sugar, and the seeds may therefore act as a safe nutrient in cases of diabetes.

These considerations suggested the importance of examining the constitution of the polysaccharide, its digestibility and effect on the blood-sugar when

ingested, also its fate in the germinated seeds, since the latter are often employed in this form. It would seem that the polysaccharide is the element in the seeds which is responsible for the alleviation of symptoms in certain medical conditions.

Experimental.

Preparation and properties of the polysaccharide.

For the preparation of this product the bran is employed, since in the milling of the seeds a considerable proportion of the hard, glassy endosperm containing the mucilage remains adherent to the outer spermoderm.

20 g. bran are mixed with about 4 litres of water in a 5-litre bottle, the mixture is shaken in a mechanical shaker for 5 hours and the contents are filtered. Filtration is generally tedious because of the viscid nature of the solution, but it can to some extent be hastened by filtration at the pump, first through calico and then through three folds of filter-paper. Alcohol up to 30 % is then added to the filtrate. A white hair-like precipitate is formed which is transferred to a suitable calico bag through which as much as possible of the fluid is squeezed out. The precipitate is dissolved in water and reprecipitated by alcohol at least three times, and the polysaccharide is finally treated with alcohol of gradually increasing concentration, followed by ether, and placed in a vacuum desiccator over calcium chloride.

The polysaccharide thus obtained is a light white solid which has no characteristic taste or smell and contains 0·83 % of ash. On drying it partly tends to form small masses which powder with difficulty. On redissolving the dry substance in water some of it passes easily into solution while the rest, which is imperfectly soluble even in boiling water, remains in solid suspension in the colloidal solution of the former and for this reason it is difficult to obtain a homogeneous solution from the dry prepared product. The moist freshly precipitated polysaccharide mixes fairly well with different proportions of cold water and it is thus possible to make it into a thick jelly with this solvent. The solutions are highly opalescent. The polysaccharide does not reduce Fehling's solution and gives no colour with iodine even after treatment with sulphuric acid. It is precipitated by basic lead acetate and also by Fehling's solution. It is soluble in hot potassium hydroxide.

The chemical composition of the polysaccharide.

The polysaccharide was hydrolysed with dilute sulphuric acid, the hydrolysate was neutralised with barium carbonate, and the filtrate was decolorised.

On treating the colourless filtrate with phenylhydrazine and acetic acid in the cold a white crystalline precipitate was obtained which was identified, by its crystalline form and its melting-point, as mannosephenylhydrazone.

On heating the mixture of phenylhydrazine and the hydrolysate and examining the osazones formed, only two could be found, one of which was

identical with glucosazone and the other with galactosazone. No osazones of the pentoses could be identified, and the absence of the latter was further confirmed by applying other chemical tests for these bodies.

That the glucosazone obtained was entirely due to the mannose, and not to the possible presence of fructose or glucose as well, was shown by applying the chemical tests for fructose to the hydrolysate which gave negative results. Glucose was proved to be absent by adding excess of phenylhydrazine dissolved in acetic acid to the cold hydrolysate and allowing the mixture to stand overnight and then filtering and heating the filtrate in a water-bath, when galactosazone only was formed.

The presence of galactose was further confirmed by preparing mucic acid both from the hydrolysate and from the unhydrolysed polysaccharide.

When the method of Nanji, Paton and Ling [1925] for testing and estimating uronic acids was applied no carbon dioxide was evolved, showing the absence of these bodies.

It is, therefore, concluded that mannose and galactose are the only sugars which enter into the constitution of this polysaccharide. The relative quantities of these two sugars were calculated as follows.

The specific rotatory power of the hydrolysate was $[\alpha]_D^{20^\circ} = 46{\cdot}2^\circ$, the total concentration of the sugars being estimated by Bertrand's method and calculated as glucose.

If X is the quantity of mannose, then $1 - X =$ the quantity of galactose. We therefore have the equation

$$14X + 81\,(1 - X) = 46{\cdot}2,$$
$$X = 0{\cdot}52.$$

So that the quantities of mannose and galactose stand in the ratio 52 : 48 or the two sugars are present in almost equal quantities.

Bourquelot and Hérissey [1900] state that the reserve carbohydrate of fenugreek seeds is a mannogalactan. When, however, the polysaccharide was ignited it left an ash which was found to contain the following elements: iron, calcium, magnesium, phosphorus and silicon. The polysaccharide contains phosphorus in organic combination and, on hydrolysis with mineral acids, a gelatinous precipitate composed mostly of silica combined with a little carbohydrate separates out. The polysaccharide is, therefore, a salt of a silicophosphoric ester of mannogalactan. The occurrence of such phosphoric and silicic esters of polysaccharides in nature has been reported by different authors; thus Pringsheim and Kusenack [1924] found that lichenin is a silicic ester, and in relation to the constitution of starch Ling and Nanji [1923] state that the amylopectin of the starch granules is a phosphoric ester, while Schryver *et al.* [1923] found that some starches contain a hemicellulose in which a certain amount of silica occurs in organic combination. Further Bourquelot and Hérissey reported that the mannogalactan of fenugreek was slightly different from that of lucerne.

From the above-mentioned description it seems that, apart from the constituent sugars, there is a close analogy in constitution between this polysaccharide and starch. The polysaccharide seems to be present in different forms and probably in different degrees of esterification with correspondingly different degrees of solubility like the amylose, amylopectin and amylohemicellulose of the starch granules.

The digestibility of the polysaccharide.

Mannogalactans are hydrolysed by the enzyme seminase into mannose and galactose. This enzyme is not known to occur in the human body. Nevertheless the action of saliva, as well as that of an extract of the pancreas on the polysaccharide was studied. In both cases a 0·5 % solution of the mucilage was used to which varying concentrations of the digestive fluids were added. The activity of these fluids was always checked by using as a control 0·5 % starch paste. Judging by Fehling's reaction, the polysaccharide appeared not to have been influenced either by saliva or by the extract of the pancreas. In looking for the presence of sugars the polysaccharide was first precipitated with alcohol, the filtrate was evaporated on a water-bath to drive off the alcohol, and the residue was then tested for reduction. This course had to be followed because of the insoluble copper compound which the polysaccharide forms on the addition of Fehling's solution and which makes the detection of a small reduction very difficult or practically impossible.

Table I.

Time	Blood-sugar %			
	Subject F.		Subject M.	
	Bran	Whole meal	Bran	Whole meal
Fasting	0·091	0·105	0·090	0·088
½ hour after ingestion of polysaccharide	0·091	0·098	0·083	0·091
1 " " "	0·095	0·098	0·090	0·091
1½ " " "	0·097	0·098	0·093	0·093
2 " " "	0·098	0·100	0·093	0·091
2½ " " "	0·091	0·103	0·092	0·094
3 " " "	—	0·093	0·088	0·086
3½ " " "	0·088	—	—	—
4 " " "	—	0·095	0·088	0·088
4½ " " "	0·088	—	—	—
5 " " "	—	0·097	0·092	0·088

This result, which was expected, suggests that the polysaccharide may be a factor in the laxative effect which is supposed to follow the ingestion of these seeds. This would undoubtedly be correct if the polysaccharide is not affected by other agencies in the alimentary canal.

As an indirect way for finding out whether the polysaccharide is hydrolysed by other agencies in the alimentary canal its effect on the blood-sugar was studied. Samples of blood were taken in the morning from two fasting men immediately before, and half-hourly after, taking 50 g. of the bran which contains more than 75 % of the polysaccharide. In other experiments 50 g. of

the whole fenugreek seed meal, which contains 50 % of the carbohydrate, were given to the same men. The method of Hagedorn and Jensen for the estimation of blood-sugar was employed in these cases. The result obtained is given in Table I. In the case of subject F. there was a very slight but continuous rise of the blood-sugar when the bran was given. This is, however, of no significance, especially when the slight fluctuations in the other experiments are taken into consideration, and it follows that if the polysaccharide is attacked at all in the alimentary canal the products have no influence on the blood-sugar. If any change takes place it would be produced by bacteria in the lower intestine.

Another important point which can be inferred from the results of giving the whole fenugreek seed meal is that, if the dry seeds contain the specific enzyme or enzymes which are capable of producing the changes which occur in the polysaccharide during the germination of the seeds, these enzymes appear to remain inactive in the alimentary canal.

The polysaccharide during germination.

As the fenugreek seeds are administered, in some cases, in the germinated form, the fate of the polysaccharide during germination and the nature of its hydrolysis products under different conditions were fully investigated. Evidence of the suitability of different sugars for performing certain functions in nature was also gained.

When the powdered ungerminated seeds are extracted with 50 % alcohol, in order to dissolve any sugars present and at the same time prevent the polysaccharide from dissolving, and the liquid is filtered and evaporated, the extract does not reduce Fehling's solution and does not form an osazone even after hydrolysis. This proves that the seeds before germination do not contain sugars. The seeds are also quite free from starch.

When, however, the seeds are allowed to germinate, sugars make their appearance. It should be remarked that the photosynthetic process does not take part in the formation of these sugars because the observations are made before the cotyledons leave the testa or show any sign of turning green, and therefore these sugars are entirely due to the hydrolysis of the reserve polysaccharide itself.

Fenugreek seeds were steeped in water for 24 hours, the water was drained off and the seeds were placed in a shallow wooden box with perforated sides, covered with moist filter-paper, placed in a fairly warm place and moistened with water from time to time. After 48 hours' germination, the radicles were completely separated from the rest of the seeds. On grinding the radicles with fine washed sand to a paste and then extracting with water and filtering, the filtrate was found to reduce Fehling's solution strongly. When a portion of the filtrate was treated with phenylhydrazine and acetic acid and heated for 1 hour glucosazone only could be identified. The absence of mannose from the extract was repeatedly confirmed by obtaining no mannosephenylhydrazone on the addition of phenylhydrazine and acetic acid in the cold. The absence of galac-

tose was further confirmed by the failure to obtain mucic acid on oxidising a portion of the extract with nitric acid. This is an interesting fact because, whilst the reserve polysaccharide is composed of galactose and mannose, these two sugars do not make their appearance in the free state among other sugars during the germination of the seeds. Observations on the endosperm indicate clearly its gradual exhaustion with the advance of germination, which proves that the polysaccharide functions as a reserve and not merely as a structural material.

As the osazone obtained can be given by fructose as well, the Pinoff-Seliwanoff [Pinoff, 1905] test was applied, using at the same time control tubes containing a dilute solution of sucrose and a dilute and a more concentrated solution of glucose. The colour developed in 1 minute in the case of the extract and the control containing sucrose, whilst in the case of the glucose solutions it took more than half an hour to develop. The positive result with Pinoff's test, together with the fact that glucosazone was the only osazone obtained, suggest the possibilities of the presence of fructose alone, glucose together with fructose, glucose together with sucrose or glucose, fructose and sucrose.

After hydrolysis, the clarified extracts of the radicles show a marked increase in reducing power, and an osazone identical with glucosazone is again the only one obtained; further, the sign of rotation is changed from positive to negative. It is clear, therefore, that sucrose is present together with the reducing sugar or sugars. The simultaneous presence of both free fructose and glucose was made evident from the result of the following experiment.

An extract of the sugars from the dried powdered radicles was prepared by adopting the method of Bryan, Given and Straughn [see Browne, 1912] for the preparation of sugar solutions from plant substances.

The specific rotatory power of the extract was found to be $[\alpha]_D^{20^\circ} + 27{\cdot}9^\circ$.

The reducing sugars, estimated by Bertrand's method and calculated as invert sugar, equal 0·485 %.

The reducing sugars, after hydrolysis calculated as invert sugar, equal 1·0675 %.

$$\therefore \text{sucrose} = (1{\cdot}0675 - 0{\cdot}485) \times 0{\cdot}95 = 0{\cdot}553\ \%.$$

It will be seen that the specific rotatory power of the extract cannot be accounted for except on the assumption that the reducing sugar consists of almost equivalent quantities of glucose and fructose, as is evident from the following calculation, considering half the reducing sugar as glucose and the other half as fructose, and multiplying the concentration of each sugar by its specific rotatory power, we obtain

$$0{\cdot}242 \times 52{\cdot}8 + (-92{\cdot}5 \times 0{\cdot}242) + 66{\cdot}5 \times 0{\cdot}55 = 27{\cdot}16.$$

Thus the calculated rotatory power agrees with that observed and it is to be concluded, therefore, that the sugars present in the radicles are sucrose, glucose and fructose. The two latter are present in almost equivalent quantities.

Galactose and mannose were also proved to be absent from the cotyledons of the germinated seeds. It is evident that these two sugars are completely transformable into glucose, fructose and sucrose during germination. It is to be expected that the formation of both glucose and fructose precedes that of sucrose since linkages between the hexose residues of the type present in sucrose would be absent from a mannogalactan. It seems that the hydrolysis of the polysaccharide and the transformation of the constituent sugars into glucose and fructose proceed at a more rapid rate than that with which the latter two sugars are utilised for the liberation of energy, and consequently sucrose is formed as a temporary reserve.

The mechanism of the transformation of mannose and galactose into glucose and fructose is obscure. As regards the former, the transformation into its ketose analogue and its epimeride may to some extent be explained by the ease with which the reversal of the stereochemical configuration of carbon atom 2 takes place, but even then the complete absence of mannose during germination suggests a different mechanism. Still more difficult to understand is the transformation of galactose. The latter is also known to take place in the human body, thus it is stated that lactose when fed to diabetics may be recovered in the urine as two molecules of glucose for each molecule of lactose fed. This indicates that the tissues can under certain conditions transform galactose into glucose. This and similar transformations have been explained by Robinson [1927] to take place through the optical inversion on the hydrolysis of a hexose-4-phosphate.

Whatever the case may be, we are faced with the difficulty of explaining the reason why this transformation takes place in the germinating seeds only and not when the hydrolysis is effected by sulphuric acid. Work on this point is still proceeding in this Department.

There are, however, other possibilities as regards the non-appearance of galactose among the other sugars during germination, such as its accumulation in a polysaccharide-like form, which may become associated with the cell walls of the newly formed cells, or its preferential utilisation for the supply of energy. To clear up the first point, the ratio of the galactose to the rest of the reducing sugar produced by hydrolysing the ungerminated and the germinated seeds by dilute sulphuric acid was studied. There is some difficulty in experimenting with the germinated seeds as the hydrolysis of these gives rise to four sugars. The best procedure was to calculate the galactose from the yield of mucic acid obtained by oxidising the neutralised hydrolysate by nitric acid and to calculate the total sugar as galactose by Fehling's solution. Although the mucic acid method for the estimation of galactose usually gives low results, it serves for comparative purposes since the same experimental conditions were exactly followed in each case. In the case of the germinated seeds precaution was taken to avoid any loss of mucilage during steeping. The seeds were weighed out and were just covered with water in a porcelain basin and, when the water was absorbed, further quantities were added; after the absorption of

the last quantity the seeds became exposed to the air and were placed in a warm place to germinate. The germinated seeds were ground up with a small quantity of washed sand and then washed into a flask.

Taking equivalent quantities of the powdered ungerminated seeds and of the seeds used for germination, all other experimental conditions being the same, the ratios of galactose in the hydrolysate to the rest of the sugar calculated as galactose were as follows:

(1) In the ungerminated seeds: galactose/the rest of the sugar = 1/2.

(2) In the germinated seeds: galactose/the rest of the sugar = 1/9.

Supposing that the galactose accumulates in some form or other and none of it is transformed, its ratio to the rest of the sugar would increase during germination as some of the sugar in the case of the germinated seeds would be utilised for the supply of energy. That the galactose is not preferentially utilised for the latter purpose is evident from the fact that it is not present in the free state in any quantity during germination.

As regards the function of the polysaccharide it is seen that the combination of galactose with mannose results in the formation of a polysaccharide which not only functions as a reserve but which also possesses the property of imbibing large quantities of water and may possibly help early growth in situations where water is not plentiful. Further, in the dry state, this polysaccharide is hard and is therefore more efficient than starch for protecting the cotyledons, which are rich in nutritive material, from attack by insects and other external influences and, in fact, these seeds keep better than starchy seeds. Glucose and fructose appear to be the only easily assimilable sugars which are utilised for the supply of energy, whilst when they are in excess they are stored in the form of a temporary local reserve in the form of sucrose.

Summary.

(1) The reserve polysaccharide of fenugreek seeds is a salt of a silicophosphoric ester of mannogalactan. The two sugars entering into its constitution are present in almost equivalent quantities.

(2) The polysaccharide is not hydrolysed by the saliva or the pancreatic extract *in vitro*, and if it is assimilated from the alimentary canal the products show no influence on the blood-sugar.

Whole fenugreek meal, like the isolated polysaccharide, does not cause a change in the blood-sugar.

(3) During the germination of the seeds the mannose and the galactose constituents of the reserve polysaccharide are transformed into glucose, fructose and sucrose. Nothing is known of the mechanism of this transformation.

In conclusion, I desire to express my thanks to Dr A. Hassan for suggesting the work and for his encouragement and advice.

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XXX. STUDIES ON THE CHEMICAL NATURE OF THE UNSAPONIFIABLE FRACTION OF FISH-LIVER OILS.

II. COD-LIVER OIL.

By the late GUNNAR WEIDEMANN.

Edited by Fredrik Ender.

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In 1926 Gunnar Weidemann published a paper, "Studies on the chemical nature of the unsaponifiable fraction of fish oil" [Weidemann, 1926], in which he describes a method for the isolation of selachyl alcohol and batyl alcohol contained in the unsaponifiable fraction of the liver oil of the Greenland shark (*Somniosus microcephalus*). Weidemann transformed the alcohols into acid esters of phthalic acid, the salts of which can be purified. This and similar methods Weidemann later used on the unsaponifiable fraction of cod-liver oil, in which he assumed four alcohols and one hydrocarbon to be present.

The untimely death of Weidemann put an end to his research work, but notes and a short description of the applied methods were preserved. On this basis I have tried to reconstruct the procedure of his work, and I shall in this paper give an account of the results which Weidemann had obtained. The difficulties especially consisted in getting a survey of the numerous fractions which, when investigated, appeared to be more or less identical. Weidemann's results seem to me to be valuable for future investigations regarding the unsaponifiable matter of cod-liver oil and the vitamins contained therein. I wish to emphasise that Weidemann himself regarded these investigations as preliminary work for future researches.

On the basis of the iodine value and the equivalent weight of the phthalic ester acids of the alcohols and from the barium content of the corresponding barium salts Weidemann calculated the formulae of these alcohols. Complete analyses, however, were not made, accordingly the formulae calculated must be regarded as hypothetical and will not be given here.

For the hydrocarbon only refractive index and iodine value are given. The analogy between two of the alcohols (G_1 and G_2) may indicate that they have not been in a pure condition, and perhaps only a single alcohol is concerned. As the hydrocarbon fraction had only been prepared as an oil, it is difficult to have any idea about its composition. Since tertiary alcohols can react with

phthalic anhydride only under special conditions [Tiemann and Krüger, 1896] such alcohols may probably be contained in the hydrocarbon fraction.

Weidemann supposes that the alcohols belong to the aliphatic and cyclic group respectively.

In the unsaponifiable fraction of cod-liver oil were found:

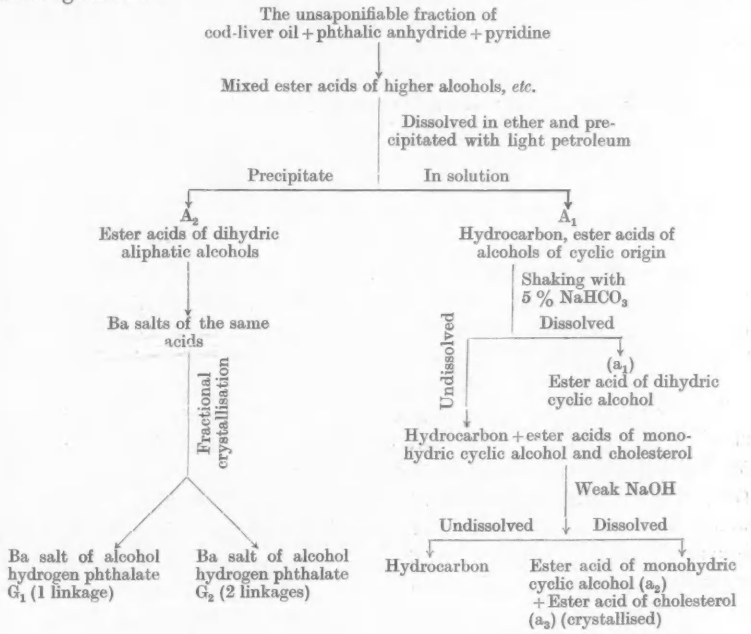
(1) A hydrocarbon differing considerably from spinacene, its refractive index being $n_D^{20^\circ} = 1{\cdot}5257$.

(2) A monohydric alcohol very sensitive to acids, and probably related to cholesterol.

(3) A dihydric alcohol.

(4) Two dihydric alcohols of the aliphatic group (G_1 and G_2).

The method of treatment of the unsaponifiable fraction is shown in the following scheme:


The unsaponifiable fraction of cod-liver oil + phthalic anhydride + pyridine
Mixed ester acids of higher alcohols, etc.
Dissolved in ether and precipitated with light petroleum
Precipitate
In solution
A2
Ester acids of dihydric aliphatic alcohols
A1
Hydrocarbon, ester acids of alcohols of cyclic origin
Shaking with 5 % NaHCO3
Dissolved
Undissolved
(a1)
Ester acid of dihydric cyclic alcohol
Ba salts of the same acids
Fractional crystallisation
Hydrocarbon + ester acids of monohydric cyclic alcohol and cholesterol
Weak NaOH
Undissolved
Dissolved
Ba salt of alcohol hydrogen phthalate G1 (1 linkage)
Ba salt of alcohol hydrogen phthalate G2 (2 linkages)
Hydrocarbon
Ester acid of monohydric cyclic alcohol (a2) + Ester acid of cholesterol (a3) (crystallised)

The unsaponifiable fraction of the oil, from which most of the cholesterol has been removed, is treated with a mixture of phthalic anhydride and pyridine, whereby the alcohols are transformed into phthalic ester acids, while the hydrocarbon and the unsaponified glycerides remain unchanged. The pyridine and the excess of phthalic anhydride are removed by shaking first with dilute sulphuric acid and then with water. A yellowish red oil remains, which contains hydrocarbon, unsaponified glycerides and phthalic ester acids of cholesterol and of unknown alcohols.

The reaction product is dissolved in ether and transferred to a separating funnel and repeatedly shaken out with fresh light petroleum. In solution are

(A_1) the hydrocarbon, glycerides and ester acids of cholesterol and the two unknown cyclic alcohols (mono- and di-hydric respectively);

(A_2) the ester acids of the dihydric aliphatic alcohols remain undissolved. The two fractions A_1 and A_2 are worked out separately.

From fraction A_1 the cyclic ester acids are isolated by shaking with an aqueous solution of sodium bicarbonate, which only dissolves the ester acid of the corresponding dihydric alcohol. By subsequent shaking with sodium hydroxide the ester acids of the monohydric alcohol and of cholesterol are dissolved. The residue of fraction A_1, consisting of glycerides and hydrocarbon, yields the hydrocarbon upon saponification.

From A_2 the phthalic esters of the two aliphatic alcohols are isolated by shaking the fraction with an aqueous solution of sodium bicarbonate, freeing the salts from bicarbonates and then precipitating with barium chloride. From the precipitated barium salts the acids are liberated with dilute mineral acid. By saponification of the acids with alcoholic potassium hydroxide the corresponding alcohols are subsequently isolated.

Experimental.

The preparation of the phthalic ester acids.

30 g. of the unsaponifiable part of the cod-liver oil still containing 16·1 % cholesterol were dissolved in 315 cc. of freshly distilled pyridine, and to this solution 66 g. of pulverised and dried phthalic anhydride were added. The solution remained for 3½ days in an opaque flask filled with nitrogen. 800 cc. of water were added and, after solution, 600 cc. of peroxide-free ether and a mixture of 210 cc. of sulphuric acid and 1000 cc. of water were added with stirring. The ether layer was removed and repeatedly washed with water until the washing water showed a neutral reaction. The ethereal solution was dried (Na_2SO_4), and evaporated in an atmosphere of carbon dioxide, and finally *in vacuo*. The residue, a yellowish red oil, weighed 50·4 g. This oil was dissolved in a small quantity of ether and then treated as indicated on p. 265. After repeated shakings of the oil with cold light petroleum the following fractions were obtained.

Fraction A_1, easily soluble in light petroleum; weight 13·4 g.

Fraction A_2, insoluble in light petroleum; weight 27·76 g.

Of these two fractions the first contains the mono- and the second the diphthalic ester acids[1].

Fraction A_1 was again esterified with phthalic anhydride according to the method previously described. The reaction product was treated with cold

[1] According to Weidemann [1926] the diphthalic ester acids differ from monophthalic ester acids in their solubility in cold light petroleum, the first being insoluble, the second easily soluble in this solvent.

light petroleum, which dissolved most of it. After evaporation of the light petroleum the soluble fraction weighed 12·20 g. It was dissolved in ether and first shaken out with a 5 % solution of sodium bicarbonate, which takes up the ester acid of the dihydric cyclic alcohol (a_1). The ether solution was then shaken with a weak sodium hydroxide solution, which dissolved the ester acid of the monohydric cyclic alcohol (a_2) and of cholesterol (a_3). From the sodium salts the ester acids themselves (a_1, a_2 and a_3) were liberated by means of dilute sulphuric acid. The cholesterol hydrogen phthalate, which is less soluble in absolute alcohol and ether than the cyclic ester acid a_2, is separated from this by crystallisation.

The ethereal solution, now free from ester acids, was evaporated and the residue saponified. The hydrocarbon remained. From the ester acids or their Ba salts respectively the alcohols were isolated in the usual way by saponification.

The following figures were found:

Ester acid a_1:
- Eq. weight ..... 353·5
- $n_D^{20°}$ ..... 1·5199
- Ba ..... 16·26 % in the Ba salt of the ester acid
- $n_D^{20°}$ ..... 1·4851 for the corresponding alcohol

This alcohol does not give the Lifschütz oxycholesterol reaction. It yields a weak blue colour with $SbCl_3$.

As to the ester acid a_2, this substance was contaminated by cholesterol hydrogen phthalate, therefore the figures found for equiv. weight, *etc.*, are not given. The alcohol contained in a_2 was prepared in a crystalline state, M.P. 144°. A negative Liebermann's reaction and a very weak Salkowski's reaction were found. With antimony trichloride a feeble blue colour appeared.

Cholesterol phthalic ester acid (a_3): Eq. weight ..... 533·1. Calculated ..... 534. M.P. ..... 162°

That this substance really was cholesterol hydrogen phthalate was proved by esterification of pure cholesterol with phthalic anhydride. The hydrogen phthalate so prepared melted at 161–161·5°. Determination of the melting-point of a mixture of the two identical ester acids has not been made.

Hydrocarbon K:
- $n_D^{20°}$ ..... 1·5257
- Iodine value ..... 180 (?)

The hydrocarbon gave no condensation product with picric acid. It gave an intense blue colour with $SbCl_3$, which was about 250 times as strong as that given by cod-liver oil and also stronger than the colour given by any of the other substances isolated.

Weidemann distilled the hydrocarbon at 0·2–0·3 mm. pressure and a temperature below 140°. The distilled hydrocarbon was a light yellowish oil. The hydrocarbon was brominated in ethereal solution, and the product treated with ether and ethyl alcohol. In this way a white substance, M.P. 164–165°, was obtained.

After having been treated with cold light petroleum, which removed a small quantity, the rest of fraction A_2 (21 g.) was dissolved in 200 cc. of ether and

shaken 3 times with a 5 % sodium bicarbonate solution in a carbon dioxide atmosphere. Only a small part, consisting of neutral products, remained in the ether solution. These products were not examined more closely. The faintly yellowish bicarbonate solution was acidified with HCl and the liberated acids were dissolved in NaOH and treated with a barium chloride solution. The precipitated barium salts were filtered and dried in a vacuum desiccator over sulphuric acid. One of the barium salts was slightly, the other easily, soluble in alcohol. Weidemann called these salts G_1Ba and G_2Ba respectively. The barium salts were shaken with 10 % HCl and ether for 1–2 hours, whereby the ester acids were liberated and taken up by the ether. The ether solution was washed, dried, and at last evaporated *in vacuo*. The residue consisted of free diphthalic ester acids[1], which were saponified at first with cold alcoholic potassium hydroxide in a nitrogen atmosphere for 24 hours, and then on a boiling water-bath for an hour. The alcohols liberated were extracted with ether. These alcohols were given the names gaddiol, gaddiol 1 and gaddiol 2 respectively.

The examination of fraction A_2 gave the following result:

G_1Ba (slightly soluble in alcohol)...18·31 % Ba.
G_2Ba (easily soluble in alcohol)...18·30 % (18·44 ?) % Ba.

The iodine values for the two alcohols were:

G_1 : 64 ?
G_2 : 160 ?

These figures are, presumably, calculated on the basis of the determination of the iodine value in the two salts G_1Ba and G_2Ba.

According to Weidemann both alcohols belong to the aliphatic group; they are, further, dihydric, a fact which can be concluded from their forming diphthalic esters.

Further researches on the unsaponifiable matter of fish-liver oils will be carried out by the present writer according to the method previously described.

In conclusion the writer wishes to thank Prof. Poulsson for helpful criticism and advice.

[1] See footnote, p. 266.

REFERENCES.

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